PA[illegible]JT COOPERATION TREAT[illegible]

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 23 April 1998 (23.04.98)	
International application No.: PCT/EP97/04744	Applicant's or agent's file reference: 15258P WO
International filing date: 01 September 1997 (01.09.97)	Priority date: 11 October 1996 (11.10.96)
Applicant: MEYER, Thomas, F. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

17 March 1998 (17.03.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

19

REC'D 27 JAN 1999
WIPO PCT

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
15258P WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP97/04744	01/09/1997	11/10/1996
International Patent Classification (IPC) or national classification and IPC		
C07K14/205		
Applicant		
MAX-PLANCK GESELLSCHAFT... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/03/1998	25.01.99
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Moonen, P Telephone No. (+49-89) 2399-8538

Form PCT/IPEA/409 (Cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP97/04744

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-48	as originally filed			

Claims, No.:

2-16	as received on	06/08/1998	with letter of	06/08/1998
1	as received on	08/01/1999	with letter of	08/01/1999

Drawings, sheets:

1/6-6/6	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.

☒ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-4, 7-15 (partially) .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-4, 7-15 (partially)
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-4, 7-15 (partially)
Industrial applicability (IA)	Yes:	Claims	1-4, 7-15 (partially)
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sh et

Reference is made to the following documents:

D1: Developments Biol Stand **84** (1995) 211-219
D2: WO 93 07273
D3: WO 96 26740
D4: WO 95 02048
D5: WO 96/34624; published 07.11.96 after the present priority date; see additionally WO 94/26901, cited in the search report.
D6: Michetti et al Gastroenterology **107** (1992) 1002-1011
D7: WO 94/03615
D8: WO 94/24291
D9: Ferrero et al PNAS **92** (1995) 6499-6503
D10: Gut **37** (1995) A51; abstract 203 (Ferrero et al.)

The documents D5-D10 were not cited in the search report. Copies were available during examination,

Item IV: Lack of unity of invention.

1. Studies in the prior art have disclosed a number of live vaccines, providing protective immunity against an infection by a pathogen (see for bacterial live vaccines D1, in particular the summary, also referring to "attenuated strains are well suited as vectors for delivery of heterologous antigenic epitopes from micro-organisms such as Helicobacter pylori..."). D1 refers to the concept of recombinant attenuated microbial pathogens transformed with DNA of H. pylori, referred to in the present application.

   D1 gives no hints or detailed instructions how to develop an efficacious **recombinant** bacterial live vaccine against Helicobacter pylori; D1 refers to the history of live bacterial vaccines. At present, D1 is therefore not considered to be an enabling disclosure, in particular in view of the last sentence of the summary of D1: "At present, the feasibility of this approach for **human** beings remains to be proven". Attention is however drawn to additional titles of lectures in session III, parallel to the lecture representing D1 (see the Contents Table, included as last page of the copy of D1), in particular to the titles "development of Bacillus

Calmette-Guérin as a live recombinant vaccine vehicle with reduced survival capacity" and "Salmonella as Oral Vaccine Carriers", and to the present description (bottom of page 2) mentioning "Advantage has been taken from the potent immunogenicity of live Salmonella vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated Salmonella has conferred murine protection against several bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing Helicobacter antigens and protecting the vaccinated animals, has not yet been described".

2. None of the other available prior art documents is considered to refer to the subject-matter of claim 1 concerning a recombinant attenuated bacterium. It appears therefore that the subject-matter of at least **claim 1 is novel,** but that it lacks an inventive step in view of D1 in combination with other available prior art like **D4** already referring in general to (recombinant) attenuated live **Salmonella** vaccine (see pages 8 and 9 of D4).

3. D2, an early patent application by Ferrero et al., has disclosed (see claim 33) as a recombinant host E. coli modified by a nucleotide sequence according to any of claims 1-12. The Helicobacter ureases, referred to in said claims 1-12, have been identified in D2 as highly relevant factors to pathogenicity, and the problem of D2 is therefore to provide means to inactivate or attenuate these enzymes (see page 2 of D2; see also D6: Michetti et al., referred to on page 1 of the present description). D2 refers also to the possibility to construct attenuated pathogens (see pages 3-4), in particular for diagnostic purposes; on page 4 first full paragraph it suggests only the use of the compositions for immunoprotection. It is therefore considered that the subject-matter of **claim 4 is novel** over the recombinant E coli defined in claims 33-34 of D2 to be an attenuated microbial pathogen. However, an inventive step is not recognisable on the basis of the disclosure of D1 in combination with the teaching of D2.

The later documents D9/D10 (all co-authored by Ferrero; see also D5 and WO 94/26901) concern a recombinant subunit vaccine to induce an immunoprotective response against gastric Helicobacter infection; D10 mentions e.g. a composition comprising a mixture of Helicobacter antigens consisting essentially of UreB and HspA of H.pylori, affording total protection against H felis infection (and provoking

a mucosal response, see claim 15 of D5). The inoculation scheme is however different from the present application; a specially important difference is the use of a toxic adjuvant (cholera holotoxin) in the work of Ferrero et al.

It is not considered that documents like D7 and D8 teach away from the suggestion of D1. The skilled person had no information that led away from the fact that an immune response was obtainable with an recombinant attenuated Salmonella (as suggested in D1).

In Summary: The prior art contained a teaching about immunoprotection (after oral immunization) by Helicobacter urease (documents D2/D6/D9/D10) **and** a suggestion about the use of a live vaccine e.g. based on Salmonella bacteria in relation to H pylori infection (see summary of D1), Salmonella referred to as an oral vaccine carrier.

4. The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT does not exist between the subject-matter of the following groups of dependent claims:

i. **Claim 4 and depending claims:** the Helicobacter antigen being urease;
ii. **Claim 5-6 and depending claims:** the antigen being a secretory polypeptide from Helicobacter like AlpA, AlpB.

5. Moreover, methods for the screening of useful antigens (epitopes) of pathogens are well known. The screening of the clones of a gene bank (e.g. see D3, page 21) for their ability to confer protective immunity against an infection in a mammalian host is usually carried out by screening for the presence of a particular antibody reactivity towards antigens. The direct testing for protective immunity is a lot of experimental work and appears in fact also not to have been carried as experimental results are missing in the specification.

An additional separate invention is therefore represented by the subject-matter of **claim 16**, as it is not so linked as to form a single general inventive concept (Rule 13.1 PCT) with the subject-matter of claims 1-15.

6. A single general inventive concept (referred to in Rule 13 PCT and the PCT Preliminary Examination Guidelines Ch.III, 7) is therefore not recognisable in the absence of a common, special technical feature, and three inventions have been identified (claim 4, 5-6 and 16, respectively). The Applicant did not pay additional fees or restrict the claims on file. The IPER is therefore be issued with respect to the first recognised invention (claim 4).

Item V: Reasoned statement under Rule 66.2(a)(ii).

7. As mentioned above it is considered that the subject-matter of **claims 1-4** is obvious to the skilled person and therefore the present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of said claims does not involve an inventive step (Rule 65(1)(2) PCT).

With respect to claim 4 in so far as it contains the combination of H pylori urease expressed in recombinant attenuated Salmonella (i.e. claim referring back to claim 3 only) an inventive step is recognised, as in this case it has been demonstrated that the problem (obtaining a protective immune response protecting agains H pylori infections) is indeed solved by expression ot the urease by living attenuated Salmonella (data both in the application as originally filed and in additional experimental data later filed) leading to full protection in mouse challenged with an H pylori strain.

The additional technical features of depending **claims 7-15** (as far as referring back to claim 4) are considered to be merely represent straightforward possibilities which the skilled person would select from several possibilities, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed. The subject-matter of said depending claims is therefore considered to be obvious to the skilled person in the absence of any demonstrated unexpected or special results.

Item VIII: Certain Observations under Article 6 PCT.

8. In conjunction with the above observation with respect to the lack of unity of invention, it is noted that Article 6 of the PCT requires that all independent claims

contain the essential technical feature(s) of the invention (see also Rule 6.3(b) PCT).

The product claims like claim 4 are now limited to products for a first medical use (pharmaceutical use). This restriction with respect to claims 1-15 is not considered to introduce all the essential technical features of the invention.

A restriction to the type of expressed Helicobacter specific antigen (urease) and carrier (attenuated Salmonella) as essential technical features of the presently examined invention appears to be necessary (e.g compare US-A-5,583,038 limited to recombinant mycobacteria). All prior art relating to succesfull immunoprotection against H pylori infection comprise urease fragments (see e.g. also abstract 204 by Ohlara on the same page A51 of cited reference D10 using a H.pylori mouse model); furthermore, success in the present application with live attenuated bacterium has only been demonstrated with recombinant attenuated Salmonella (in mice), and the skilled person has no teaching or expectation that he may equally use **any** other attenuated bacterium to be equally succesfull (see also the summary of D1).

****

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WEICKMANN WEICKMANN PRECHTEL WEISS
TIESMEYER HERZOG BÖHM LISKA & HUBER
Kopernikusstrasse 9
D-81679 München
ALLEMAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	25. 01. 99
Applicant's or agent's file reference 15258P WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP97/04744	01/09/1997	11/10/1996

Applicant
MAX-PLANCK GESELLSCHAFT... et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Vullo, C Tel. (+49-89) 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
15258P WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP97/04744	01/09/1997	11/10/1996
International Patent Classification (IPC) or national classification and IPC		
C07K14/205		
Applicant		
MAX-PLANCK GESELLSCHAFT... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/03/1998	25. 01. 99
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Moonen, P Telephone No. (+49-89) 2399-8538

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP97/04744

I. Basis f th report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-48	as originally filed			

Claims, No.:

2-16	as received on	06/08/1998	with letter of	06/08/1998
1	as received on	08/01/1999	with letter of	08/01/1999

Drawings, sheets:

1/6-6/6	as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☒ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☐ all parts.

☒ the parts relating to claims Nos. 1-4, 7-15 (partially) .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-4, 7-15 (partially)
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-4, 7-15 (partially)
Industrial applicability (IA)	Yes:	Claims	1-4, 7-15 (partially)
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see s parat sheet

Reference is made to the following documents:

D1: Developments Biol Stand **84** (1995) 211-219
D2: WO 93 07273
D3: WO 96 26740
D4: WO 95 02048
D5: WO 96/34624; published 07.11.96 after the present priority date; see additionally WO 94/26901, cited in the search report.
D6: Michetti et al Gastroenterology **107** (1992) 1002-1011
D7: WO 94/03615
D8: WO 94/24291
D9: Ferrero et al PNAS **92** (1995) 6499-6503
D10: Gut **37** (1995) A51; abstract 203 (Ferrero et al.)

The documents D5-D10 were not cited in the search report. Copies were available during examination,

Item IV: Lack of unity of invention.

1. Studies in the prior art have disclosed a number of live vaccines, providing protective immunity against an infection by a pathogen (see for bacterial live vaccines D1, in particular the summary, also referring to "attenuated strains are well suited as vectors for delivery of heterologous antigenic epitopes from micro-organisms such as Helicobacter pylori..."). D1 refers to the concept of recombinant attenuated microbial pathogens transformed with DNA of H. pylori, referred to in the present application.

   D1 gives no hints or detailed instructions how to develop an efficacious **recombinant** bacterial live vaccine against Helicobacter pylori; D1 refers to the history of live bacterial vaccines. At present, D1 is therefore not considered to be an enabling disclosure, in particular in view of the last sentence of the summary of D1: "At present, the feasibility of this approach for **human** beings remains to be proven". Attention is however drawn to additional titles of lectures in session III, parallel to the lecture representing D1 (see the Contents Table, included as last page of the copy of D1), in particular to the titles "development of Bacillus

Calmette-Guérin as a live recombinant vaccine vehicle with reduced survival capacity" and "Salmonella as Oral Vaccine Carriers", and to the present description (bottom of page 2) mentioning "Advantage has been taken from the potent immunogenicity of live Salmonella vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated Salmonella has conferred murine protection against several bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing Helicobacter antigens and protecting the vaccinated animals, has not yet been described".

2. None of the other available prior art documents is considered to refer to the subject-matter of claim 1 concerning a recombinant attenuated bacterium. It appears therefore that the subject-matter of at least **claim 1 is novel,** but that it lacks an inventive step in view of D1 in combination with other available prior art like **D4** already referring in general to (recombinant) attenuated live **Salmonella** vaccine (see pages 8 and 9 of D4).

3. D2, an early patent application by Ferrero et al., has disclosed (see claim 33) as a recombinant host E. coli modified by a nucleotide sequence according to any of claims 1-12. The Helicobacter ureases, referred to in said claims 1-12, have been identified in D2 as highly relevant factors to pathogenicity, and the problem of D2 is therefore to provide means to inactivate or attenuate these enzymes (see page 2 of D2; see also D6: Michetti et al., referred to on page 1 of the present description). D2 refers also to the possibility to construct attenuated pathogens (see pages 3-4), in particular for diagnostic purposes; on page 4 first full paragraph it suggests only the use of the compositions for immunoprotection. It is therefore considered that the subject-matter of **claim 4 is novel** over the recombinant E coli defined in claims 33-34 of D2 to be an attenuated microbial pathogen. However, an inventive step is not recognisable on the basis of the disclosure of D1 in combination with the teaching of D2.

The later documents D9/D10 (all co-authored by Ferrero; see also D5 and WO 94/26901) concern a recombinant subunit vaccine to induce an immunoprotective response against gastric Helicobacter infection; D10 mentions e.g. a composition comprising a mixture of Helicobacter antigens consisting essentially of UreB and HspA of H.pylori, affording total protection against H felis infection (and provoking

a mucosal response, see claim 15 of D5). The inoculation scheme is however different from the present application; a specially important difference is the use of a toxic adjuvant (cholera holotoxin) in the work of Ferrero et al.

It is not considered that documents like D7 and D8 teach away from the suggestion of D1. The skilled person had no information that led away from the fact that an immune response was obtainable with an recombinant attenuated Salmonella (as suggested in D1).

In Summary: The prior art contained a teaching about immunoprotection (after oral immunization) by Helicobacter urease (documents D2/D6/D9/D10) **and** a suggestion about the use of a live vaccine e.g. based on Salmonella bacteria in relation to H pylori infection (see summary of D1), Salmonella referred to as an oral vaccine carrier.

4. The requisite unity of invention (Rule 13.1 PCT) therefore no longer exists inasmuch as a technical relationship involving one or more of the same or corresponding special technical features in the sense of Rule 13.2 PCT does not exist between the subject-matter of the following groups of dependent claims:

i. **Claim 4 and depending claims:** the Helicobacter antigen being urease;
ii. **Claim 5-6 and depending claims:** the antigen being a secretory polypeptide from Helicobacter like AlpA, AlpB.

5. Moreover, methods for the screening of useful antigens (epitopes) of pathogens are well known. The screening of the clones of a gene bank (e.g. see D3, page 21) for their ability to confer protective immunity against an infection in a mammalian host is usually carried out by screening for the presence of a particular antibody reactivity towards antigens. The direct testing for protective immunity is a lot of experimental work and appears in fact also not to have been carried as experimental results are missing in the specification.

An additional separate invention is therefore represented by the subject-matter of **claim 16**, as it is not so linked as to form a single general inventive concept (Rule 13.1 PCT) with the subject-matter of claims 1-15.

6. A single general inventive concept (referred to in Rule 13 PCT and the PCT Preliminary Examination Guidelines Ch.III, 7) is therefore not recognisable in the absence of a common, special technical feature, and three inventions have been identified (claim 4, 5-6 and 16, respectively). The Applicant did not pay additional fees or restrict the claims on file. The IPER is therefore be issued with respect to the first recognised invention (claim 4).

Item V: Reasoned statement under Rule 66.2(a)(ii).

7. As mentioned above it is considered that the subject-matter of **claims 1-4** is obvious to the skilled person and therefore the present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of said claims does not involve an inventive step (Rule 65(1)(2) PCT).

With respect to claim 4 in so far as it contains the combination of H pylori urease expressed in recombinant attenuated Salmonella (i.e. claim referring back to claim 3 only) an inventive step is recognised, as in this case it has been demonstrated that the problem (obtaining a protective immune response protecting agains H pylori infections) is indeed solved by expression ot the urease by living attenuated Salmonella (data both in the application as originally filed and in additional experimental data later filed) leading to full protection in mouse challenged with an H pylori strain.

The additional technical features of depending **claims 7-15** (as far as referring back to claim 4) are considered to be merely represent straightforward possibilities which the skilled person would select from several possibilities, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed. The subject-matter of said depending claims is therefore considered to be obvious to the skilled person in the absence of any demonstrated unexpected or special results.

Item VIII: Certain Observations under Article 6 PCT.

8. In conjunction with the above observation with respect to the lack of unity of invention, it is noted that Article 6 of the PCT requires that all independent claims

contain the essential technical feature(s) of the invention (see also Rule 6.3(b) PCT).

The product claims like claim 4 are now limited to products for a first medical use (pharmaceutical use). This restriction with respect to claims 1-15 is not considered to introduce all the essential technical features of the invention.

A restriction to the type of expressed Helicobacter specific antigen (urease) and carrier (attenuated Salmonella) as essential technical features of the presently examined invention appears to be necessary (e.g compare US-A-5,583,038 limited to recombinant mycobacteria). All prior art relating to succesfull immunoprotection against H pylori infection comprise urease fragments (see e.g. also abstract 204 by Ohlara on the same page A51 of cited reference D10 using a H.pylori mouse model); furthermore, success in the present application with live attenuated bacterium has only been demonstrated with recombinant attenuated Salmonella (in mice), and the skilled person has no teaching or expectation that he may equally use **any** other attenuated bacterium to be equally succesfull (see also the summary of D1).

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Claims

1. Pharmaceutical composition comprising as an active agent an immunologically protective living vaccine which is a recombinant attenuated microbial pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable of expressing said nucleic acid molecule or capable of causing the expression of said nucleic acid molecule in a target cell.

2. The composition according to claim 1, wherein the pathogen is an enterobacterial cell, especially a Salmonella cell.

3. The composition according to claim 1 or 2, wherein the pathogen is a Salmonella aro mutant cell.

4. The composition according to any of claims 1-3, wherein the Helicobacter antigen is urease, a urease subunit, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

5. The composition according to any one of claims 1-3, wherein the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

6. The composition according to any one of claims 1-3 and 5, wherein the Helicobacter antigen is selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive fragments thereof, or a peptide mimotope thereof.

7. The composition according to any one of claims 1-6, wherein said nucleic acid molecule encoding a Helicobacter antigen is capable to be expressed phase variably.

8. The composition according to claim 7,

wherein said nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal which is substantially inactive in the pathogen and which is capable to be activated by a nucleic acid reorganization caused by a nucleic acid reorganization mechanism in the pathogen.

9. The composition according to claim 8, wherein the expression signal is a bacteriophage promoter, and the activation is caused by a DNA reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

10. The composition according to any one of claims 1-9, wherein said pathogen further comprises at least one second nucleic acid molecule encoding an immunomodulatory polypeptide, wherein said pathogen is capable to express said second nucleic acid molecule.

11. The composition according to any one of claims 1-10, together with pharmaceutically acceptable diluents, carriers and adjuvants.

12. The composition according to claim 11, which is suitable for administration to a mucosal surface or via the parenteral route.

13. A method for the preparation of a living vaccine comprising formulating a pharmaceutical composition according to any one of claims 1-10 in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

14. The method of claim 13 including the preparation of a recombinant attenuated pathogen comprising the steps:

a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein a recombinant attenuated pathogen is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell, and

b) cultivating said recombinant attenuated pathogen under suitable conditions.

15. The method according to claim 14, wherein said nucleic acid molecule encoding a Helicobacter antigen is located on an extrachromosomal plasmid or inserted in the chromosome.

16. A method for identifying Helicobacter antigens, which raise a protective immune response in a mammalian host, comprising the steps of:

a) providing an expression gene bank of Helicobacter in an attenuated pathogen and

b) screening the clones of the gene bank for their ability to confer protective immunity against a Helicobacter infection in a mammalian host.

New Claims

1. Pharmaceutical composition comprising as an active agent an immunologically protective living vaccine which is a recombinant attenuated bacterium which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable of expressing said nucleic acid molecule or capable of causing the expression of said nucleic acid molecule in a target cell.

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(57) Abstract

The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by Helicobacter pylori and a method of screening H. pylori antigens for optimized vaccines.

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Helicobacter pylori live vaccine

Specification

The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by Helicobacter pylori and a method of screening H. pylori antigens for optimized vaccines.

Helicobacter is a gram-negative bacterial pathogen associated with the development of gastritis, pepic ulceration and gastric carcinoma. Several Helicobacter species colonize the stomach, most notably H. pylori, H. heilmanii and H. felis. Although H. pylori is the species most commonly associated with human infection, H. heilmanii and H. felis also have been found to infect humans. High H. pylori infection rates are observed in third world countries, as well as in industrialized countries. Among all the virulence factors described in H. pylori, urease is known to be essential for colonisation of gnobiotic pigs and nude mice. Urease is an enzyme composed of two structural subunits (UreA and UreB). Previous studies have indicated that oral immunization using recombinant UreB plus cholera toxin were able to protect mice from gastric colonisation with H. felis and H. pylori (Michetti et al., Gastroenterology 107 (1994), 1002-1011). By oral administration of recombinant UreB antigens, however, in several cases only an incomplete protection can be obtained. Other H. pylori antigens shown to give partial protection are the 87 kD vacuolar cytotoxin VacA (Cover and Blaser, J. Biol. Chem. 267 (1992), 10570; Marchetti et al., Science 267 (1995), 1655) and the 13 and 58 kD heat shock proteins HspA and HspB (Ferrero et al., Proc. Natl. Acad. Sci. USA 92 (1995), 6499).

Attenuated pathogens, e.g. bacteria, such as Salmonella, are known to be efficient live vaccines. The first indications of the efficacy of attenuated Salmonella as good vaccine in hu-

mans came from studies using a chemically mutagenized Salmonella typhi Ty21a strain (Germanier and Furer, J. Infect. Dis. 141 (1975), 553-558), tested successfully in adult volunteers (Gilman et al., J. Infect. Dis. 136 (1977), 717-723) and later on in children in a large field trial in Egypt (Whadan et al., J. Infect. Dis. 145 (1982), 292-295). The orally administered Ty21a vaccine was able to protect 96% of the Egyptian children vaccinated during three years of surveillance. Since that time new attenuated Salomonella live vector vaccines have developed (Hone et al., Vaccine 9 (1991), 810-816), in which well defined mutations incorporated into the chromosome gave rise to non-virulent strains able to induce strong immune responses after oral administration (Tacket et al., Vaccine 10 (1992), 443-446 and Tacket et al., Infect. Immun. 60 (1992), 536-541). Other advantages of the live attenuated Salmonella vaccine include its safety, easy administration, long-time protection and no adverse reactions in comparison with the former inactivated wholesale typhoid vaccines (Levine et al., Typhoid Fever Vaccines. In: Plotkin S.A., Mortimer E.A. Jr. (eds.) Vaccines. Philadelphia: WB Saunders (1988), 333-361).

Mutants of S. typhimurium have been extensively used to deliver antigens because of the possibility to use mice as an animal model, which is believed to mimick S. typhi infections in humans. The attenuation of S. typhimurium most commonly used consists in site directed mutagenesis of genes affecting the synthesis of aromatic amino acids. Such strains, designated aro mutants, have a negligible pathogenicity, as demonstrated in animal models and human trials using these constructs (Hoiseth and Stocker, Nature 291 (1981), 238-239; Tacket et al. (1992), Supra). Advantage has been taken from the potent immunogenicity of live Salmonella vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated Salmonella has conferred murine protection against several bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing Helicobacter antigens and protecting the vaccinated animals, has not yet been described.

The use of attenuated live vaccines for the treatment of a Helicobacter infection has also not been rendered obvious. The reason therefor being that in the course of the Helicobacter infection a strong immune response against the pathogen per se is induced, which, however, does not lead to a protective immunity. Thus, it was highly surprising that a protective immune response is achieved when using recombinant attenuated bacterial cells as antigen carriers, which are capable of expressing a DNA molecule encoding a Helicobacter antigen. Apparently, recombinant attenuated bacterial cells expressing a Helicobacter antigen are capable of creating a qualitatively different immune response against the heterologous Helicobacter antigen than Helicobacter itself does against its own homologous antigen. Surprisingly, a non-protective immune response is thus transformed into an immune response protecting against Helicobacter infections. This unexpected observation renders it possible to use recombinant attenuated pathogens, e.g. bacterial cells, particularly Salmonella, as carriers for the screening of protective antigens, to apply the protective antigens identified in this manner in any vaccine against Helicobacter infections, and to use recombinant attenuated bacteria as carriers of protective antigens for the immunization against Helicobacter infections in humans and other mammals.

Thus, a subject matter of the present invention is a recombinant attenuated pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable to express said nucleic acid molecule or capable to cause the expression of said nucleic acid in a target cell. Preferably the nucleic acid molecule is a DNA molecule.

The attenuated pathogen is a microorganism strain which is able to cause infection and preferably effective immunological protection against the actual pathogen but is no longer pathogenic per se. The attenuated pathogen can be a bacterium, a

virus, a fungus or a parasite. Preferably it is a bacterium, e.g. Salmonella, such as S. typhimurium or S. typhi, Vibrio cholerae (Mekalanos et al., Nature 306 (1983), 551-557), Shigella Species such as S. flexneri (Sizemore et al., Science 270 (1995), 299-302; Mounier et al., EMBO J. 11 (1992), 1991-1999), Listeria such as L. monocytogenes (Milon and Cossart, Trends in Microbiology 3 (1995), 451-453), Escherichia coli, Streptococcus, such as S. gordonii (Medaglini et al., Proc. Natl. Acad. Sci. USA 92 (1995) 6868-6872) or Mycobacterium, such as Bacille Calmette Guerin (Flynn, Cell. Mol. Biol. 40 Suppl. 1 (1994), 31-36). More preferably the pathogen is an attenuated enterobacterium such as Vibrio cholerae, Shigella flexneri, Escherichia coli or Salmonella. Most preferably the attenuated pathogen is a Salmonella cell, e.g. a Salmonella aro mutant cell. The attenuated pathogen, however, can be a virus, e.g. an attenuated vaccinia virus, adenovirus or pox virus.

The nucleic acid molecule which is inserted into the pathogen codes for a Helicobacter antigen, preferably a H. felis, H. heilmanii or H. pylori antigen, more preferably a H. pylori antigen. The Helicobacter antigen can be a native Helicobacter polypeptide, an immunologically reactive fragment thereof, or an immunologically reactive variant of a native polypeptide or of a fragment thereof. Further, the Helicobacter antigen can be a protective carbohydrate or a peptide mimotope simulating the three-dimensional structure of a native Helicobacter antigen. Peptide mimotopes can be obtained from peptide libraries presented on the surface of bacterial cells (cf. PCT/EP96/01130). Of course, the transformed cell can also contain several DNA molecules coding for different Helicobacter antigens.

The nucleic acid molecules coding for Heliobacter antigens may be located on an extrachromosomal vector, e.g. a plasmid, and/or integrated in the cellular chromosome of the pathogen. When the pathogen is used as a vaccine, chromosomal integra-

tion usually is preferred.

Attenuated bacteria can be used to transcribe and translate said nucleic acid molecule directly in the bacterial cell or to deliver said nucleic acid molecule to the infected target cell, such that the DNA molecule is transcribed and/or translated by the eukaryotic target cell machinery. This indirect bacterial vaccination procedure, termed here as genetic vaccination, has been successfully used with Shigella as a carrier (Sizemore, D. R., Branstrom, A. A. & Sadoff, J. C. (1995) Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. Science 270:299-302).

In a preferred embodiment of the present invention the Helicobacter antigen is urease, a urease subunit or an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. In a further preferred embodiment of the present invention the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. A process for identifying Helicobacter genes coding for such secretory polypeptides, and particularly for adhesins, has been disclosed in the international patent application PCT/EP96/02544, which is incorporated herein by reference. This process comprises

a) preparing a gene bank of H. pylori DNA in a host organism containing an inducible transposon coupled to a marker of secretory activity,

b) inducing the insertion of the transposon into the H. pylori DNA and

c) conducting a selection for clones containing a secretory gene by means of the marker, and optionally further

d) conducting a retransformation of H. pylori by means of the DNA of clones containing genes having secretory activity, wherein isogenic H. pylori mutant strains are produced by means of integrating the DNA into the chromosome, and

e) conducting a selection detecting adherence-deficient H. pylori mutant strains.

Suitable examples of antigens obtainable by the above process are selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive variants or fragments thereof or peptide mimotopes thereof. The nucleic and amino acid sequences of the antigens AlpA and AlpB have been disclosed in the international patent applications PCT/EP96/02545 and PCT/-EP96/04124, which are incorporated herein by reference. Further, the nucleic and amino acid sequences of AlpB are shown in SEQ ID N0. 1 and 2, and the nucleic and amino acid sequences of AlpA in SEQ ID N0. 3 and 4.

It is also conceivable, however, that an intracellular antigen is used which can be presented on the surface, e.g. by autolytic release, and confers immunological protection.

The presentation of the Helicobacter antigens in the recombinant pathogen according to the invention can be accomplished in different ways. The antigen or the antigens can be synthesized in a constitutive, inducible or phase variable manner in the recombinant pathogen. Concerning the constitutive or inducible synthesis of the Helicobacter antigens known expression systems can be referred to, as have been described by Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press.

Particularly preferred the antigens are presented in a phase variable expression system. Such a phase variable expression system for the production and presentation of foreign antigens in hybrid live vaccines is disclosed in EP-B-0 565 548, which is herein incorporated by reference. In such a phase variable expression system the nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal, which is substantially inactive in the pathogen, and which is capable of being activated by a spontaneous reorganization

caused by a nucleic acid, e.g. DNA reorganization mechanism in the pathogen, e.g. a specific DNA inversion process, a specific DNA deletion process, a specific DNA replication process or a specific slipped-strand-mispairing mechanism.

A recombinant cell having a phase variable expression system is capable of forming two subpopulations A and B, wherein the division into said subpopulations occurs by spontaneous reorganization in the recombinant nucleic acid, wherein said subpopulation A is capable of infection and immunologically active per se, while subpopulation B, which is regenerated from subpopulation A, produces at least one heterologous Helicobacter antigen and acts immunologically with respect to said additional antigen.

The activation of the expression signal encoding the Helicobacter antigen can be directly accomplished by nucleic acid reorganization or, alternatively, indirectly accomplished by activation of a gene encoding a protein which controls the expression of the gene encoding the Helicobacter antigen. The indirect activation represents a system which allows the production of the Helicobacter antigen via a cascade system, which can be realized e.g. in that the gene directly controlled by DNA reorganization codes for an RNA polymerase which is specific for the promoter preceding the Helicobacter gene, or a gene regulator which in another specific manner induces the expression of the Helicobacter gene. In an especially preferred embodiment of the present invention the expression signal for the gene encoding the Helicobacter antigen is a bacteriophage promoter, e.g. a T3, T7 or SP6 promoter, and the activation of the expression signal is caused by a nucleic acid reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

The phase variable expression system can be adjusted to provide a preselected expression level of the Helicobacter antigen. This can be accomplished e.g. by modifying the nucleotide

sequence of the expression signal, which is activated by the nucleic acid reorganization mechanism, and/or by inserting further genetic regulation elements.

The Helicobacter antigens can be produced in an intracellular, as well as in an extracellular manner in the pathogen according to the invention. For instance, autotransporter systems such as the IgA-protease system (cf. for instance EP-A-0 254 090) or the E. coli AIDA-1 adhesin system (Benz et al., Mol. Microbiol. 6 (1992), 1539) are suited as extracellular secretory system. Other suitable outer membrane transporter systems are the RTX-toxin transporters, e.g. the E. coli hemolysin transport system (Hess et al., Proc. Natl. Acad. Sci. USA 93 (1996), 11458-11463).

The pathogen according to the invention can contain a second heterologous nucleic acid, e.g. DNA molecule, which codes for an immunomodulatory polypeptide influencing the immune response quantitatively or qualitatively, apart from the nucleic acid molecule encoding the Helicobacter antigen. Examples of such immunomodulatory polypeptides are immune-stimulating peptides, cytokines like IL-2, IL-6 or IL-12, chemokines, toxins, such as cholera toxin B or adhesins.

The present invention also refers to a pharmaceutical composition comprising as an active agent a recombinant attenuated pathogen as described above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants. Preferably, the composition is a living vaccine. The vaccination routes depend upon the choice of the vaccination vector. The administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself, or the route of administration. Usually the dosage comprises about 10^6 to 10^{12} cells (CFU), preferably about 10^8 to 10^{10} cells (CFU) per vaccination. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract)

or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen. A method for the preparation of the living vaccine comprises formulating the attenuated pathogen in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

The pharmaceutical composition may be provided in any suitable form, e.g. a suspension in suitable liquid carrier, such as water or milk, a capsule, a tablet etc. In a preferred embodiment of the present invention the composition is a lyophilized product which is suspended in a liquid carrier prior to use.

Further, the present invention refers to a method for preparing a recombinant attenuated pathogen as defined above, comprising the steps of a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein the recombinant pathogen, e.g. a transformed bacterial cell, is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell and b) cultivating said recombinant attenuated pathogen under suitable conditions. If the pathogen is a bacterial cell, the nucleic acid molecule encoding the Helicobacter antigen can be located on an extrachromosomal plasmid. It is, however, also possible to insert the nucleic acid molecule into the chromosome of the pathogen.

Furthermore, the present invention refers to a method for identifying Helicobacter antigens which raise a protective immune response in a mammalian host, comprising the steps of: a) providing an expression gene bank of Helicobacter in an attenuated pathogen and b) screening the clones of the gene bank for the ability to confer a protective immunity against a Helicobacter infection in a mammalian host. Preferably, this identification process takes place in a phase variable expression system, rendering possible a stable expression of all of

the Helicobacter antigens. Recombinant clones can then be applied as "pools" for the oral immunization of test animals, such as mice. The potential of these clones as protective antigen is then determined via a challenge infection with Helicobacter, e.g. a mouse-adapted H. pylori strain. Thus, there is a possibility of directly selecting optimized H. pylori vaccine antigens.

The invention will be further illustrated by the following figures and sequence listings.

Fig. 1: shows a schematic illustration of the urease expression vector pYZ97, whereon the genes coding for the urease subunits UreA and UreB are located under transcriptional control of the T7 promoter $\phi 10$. There is a ribosomal binding site (RBS) between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (ori), a ß-lactamase resistance gene (bla) and 4 T7 terminators in series.

Apart from the expression by the T7 promoter, a constitutive low level expression of the urease A and B subunits can also be brought about via a cryptic promoter, which is located upstream from the T7 promoter, on the plasmid pYZ97.

Fig.2: shows the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Fig.3: shows a schematic illustration of the T7 RNA polymerase (T7RNAP) expression cassettes pYZ88, pYZ84 and pYZ114, which can be integrated into the chromosomes of bacteria.

In the high-expression cassette pYZ88 the lambda PL promoter is located in inverse orientation, upstream from the T7RNAP gene. A gene for the temperature-sensitive repressor cI 857 (cI) is under control of this promoter. A terminator of the bacteriophage fd (fdT) is situated upstream from the cI gene. The gin gene (Mertens, EMBO J. 3 (1984), 2415-2421) codes for a control enzyme of a DNA reorganization mechanism. A DNA sequence coding for the tRNA Arg is located downstream from the gin gene.

In phase A the PL promoter responsible for the expression of the T7RNAP gene is directed in the direction of the cI857 gene and the gin gene. The consequence of this is that an active repressor is formed at the permissive temperature of 28°C and reduces the transcription from the PL promoter. At a higher temperature the transcription of the PL promoter is increased, since the repressor is inactivated at least partially under such external influences. The temperature-dependent increase in the transcription also causes a corresponding increase in the expression of the following gin gene, which as a control enzyme catalyses the inversion of the PL promoter and the transition in phase B, in which the T7RNAP gene is expressed.

In the high-expression system pYZ88 a further fdT transcription terminator is located between a kanamycin-resistance gene (km) and the promoter of this gene. In this manner, the synthesis of an anti-sense RNA, inversely orientated to the T7RNAP gene, which normally contributes to the reduction of the T7RNAP expression, is reduced. This results in a high expression of the T7RNAP.

In the medium-expression system pYZ84 a transcrip-

tion terminator (fdT) is located between the PL promoter and the start of the T7RNAP gene. In this manner the expression of the T7RNAP mRNA is reduced. Additionally, the anti-sense RNA affects the T7RNAP translation. Therefore, only a medium expression occurs.

In the low-expression system pYZ114 a deletion of 100 bp in PL is additionally introduced (Δ PL). In this manner the activity of the PL promoter is reduced to a high extent, which leads to a lower T7RNAP expression and thus to a reduction of the UreA/B gene expression. In this construct the effect of the cryptic promoter on pYZ97 is already observed.

Fig.4: shows the results of an ELISA for anti-H.pylori antibodies in intestinal fluids of vaccinated mice.

Fig.5: shows the results of an ELISA for anti-H.pylori antibodies in the serum of vaccinated mice.

Fig.6: shows the urease activity in the stomach tissue of vaccinated mice after H.pyroli challenge.

SEQ ID NO. 1 and 2 show the nucleotide sequence of the adhesin gene AlpB from H. pylori and the amino acid sequence of the polypeptide coded therefrom.

SEQ ID N0. 3 and 4 show the nucleotide sequence of the adhesin gene AlpA from H. pylori and the amino acid sequence of the protein coded therefrom.

SEQ ID NO. 5 and 6 show the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Experimental part

Example 1

Cloning of the *ureA* and *ureB* genes.

The structural genes encoding the urease, *ureA* and *ureB*, have been genetically cloned from chromosomal DNA of a clinical specimen P1 (formerly 69A) isolated at the University of Amsterdam and provided by Dr. Jos van Putten. The genes were isolated by a PCR-approach using the primer pair YZ019 (5´-GGAATTCCATATGAAACTGACTCCCAAAGAG-3´) and RH132 (5´-CTGCAGTCGACTAGAAAATGCTAAAGAG-3´) for amplification. The sequence of the primers was deduced from GenBank (accession numbers M60398, X57132). The DNA sequence of primer YZ019 covered the nucleotides 2659-2679 of the published sequence and further contained a translational regulatory sequence (down stream box; Sprengart, M. L. et al., 1990, Nuc. Acid. Res. 18:1719-1723) and a cleavage site for *NdeI*. The DNA sequence of primer RH132 covered the nucleotides 5071-5088 of the published sequence and a cleavage site for *SalI*. The amplification product was 2.4 kbp in size comprising the complete coding region of *ureA* and *ureB* genes without the original transcriptional start and termination sequences from the *Helicobacter* chromosome. The purified PCR-fragment was digested with *NdeI* and *SalI* and inserted into the corresponding cloning sites of T7 expression plasmid pYZ57 to yield the plasmid pYZ97.

pYZ57 was originally derived from plasmid pT7-7, which was described by Tabor (1990, *In* Current Protocols in Molecular Biology, 16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York). Two terminator fragments were introduced into the pT7-7 backbone at different sites by the following strategy: (1) The tandem T7 terminators. A 2.2 kbp *EcoRI/HindIII* fragment was excised from pEP12 (Brunschwig & Darzins, 1992, Gene, 111:35-41) and the purified fragment ligated with predigested pBA (Mauer, J. et al., 1997, J. Bacteriol. 179:794-804). The ligation product was digested

with *HindIII* and *ClaI*. The resulting 2.2 kbp *HindIII/ClaI* fragment was subsequently inserted into predigested pT7-7. (2) The T1 terminator. A 230 bp *HpaI/NdeI*-fragment was excised from plasmid pDS3EcoRV (provided by Dr. H. Bujard; ZMBH, Heidelberg). The fragment was then further treated with *Klenow* to generate blunt ends. The purified rrnBT1 fragment was inserted into the previous pT7-7 derivative, predigested with *BglII* and subsequently bluntended by Klenow treatment. Figure 1 describes the completed vector pYZ97 used for the expression of the urease genes coding for urease subunits UreA and UreB in *S. typhimurium*. As indicated in Figure 1, the urease genes can be controlled by the T7 promoter ϕ10. The ribosome binding site (RBS) is located between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (*ori*) and a ß-lactamase resistance gene (*bla*).

Apart from the expression controled by the T7 promoter, a constitutive moderate level expression of the urease A and B subunits does occur from a promoter driven by *Salmonella* RNA polymerase. The promoter is located upstream from the T7 promoter, on the plasmid pYZ97. For detailed molecular analysis, the purified *BglII/HindIII*-fragment of pYZ97 was subcloned into the pCR-Script™ SK(+)kit (Stratagene) and subjected to DNA-sequencing. The sequence data confirmed the various elements in their completeness (see Figure 2 and SEQ ID NO.5 and 6): part of the *ureA* gene, the down-stream box, the RBS, the T7 promoter and the T1 terminator (rrnBT1). The sequence analysis also disclosed the region between the T1 terminator region and the T7 promoter where the *Salmonella* RNA polymerase promoter is localised. The sequence data suggests a location of this constitutive promoter between nucleotides 222 - 245 which have been deduced from structural predictions (Lisser & Margalit, 1993, Nuc. Acid. Res. 21:1507-1516).

Example 2

Immunological protection by administration of live vaccine

Materials and Methods

Bacterial strains: S. typhimurium SL3261 live vector vaccine strain was used as a recipient for the recombinant H. pylori urease plasmid constructs. S. typhimurium SL3261 is an aroA transposon mutant derived from S. typhimurium SL1344 wild type strain. S. typhimurium SL3261 is a non-virulent strain that gives protection to mice against infection with wild type S. typhimurium after oral administration (Hoiseth and Stocker (1981) Supra). S. typhimurium SL3261 and derivatives thereof, which contain the urease expression plasmid pYZ97 (extrachromosomal) and the T7RNAP expression cassettes pYZ88, pYZ84 or pYZ114, respectively (integrated into the chromosome) are indicated in table 1. Luria broth or agar was used for bacterial growth at 28°C. H. pylori wild type strain grown at 37°C on serum plates was used for the challenge experiments.

Immunization of mice: Four weeks Balb/c mice purchased from Interfauna (Tuttlingen, Germany) were adapted two weeks in an animal facility before being used for experimentation. 150 µl of blood was taken retroorbitally from all mice to obtain preimmune serum. Retroorbital bleedings were repeated from all immunized mice 1 week and 3 weeks after immunization.

Eight groups of 5 mice including controls were used in this study (table 2). Group A, the naive control group, was not immunized with Salmonella neither challenged with wild type H. pylori. The rest of the groups were all orally immunized. Group B, a negative control group, did not receive Salmonella and was challenged with H. pylori. Mice from groups C to G were immunized with Salmonella vaccine strains and challenged with H. pylori. The last group H received recombinant urease B in combination with cholera toxin and was also challenged.

Prior to immunizations mice were left overnight without solid food and 4 hours without water. 100 µl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Then mice from group B received 100 µl PBS and mice from groups C to G received 1.0×10^{10} CFU

of Salmonella in a 100 µl volume. Mice from group H received four times 100 µl of a mixture of recombinant H. pylori UreaseB plus cholera toxin, one dose every week. After every immunization water and food were returned to the mice.

H. pylori challenge: Four weeks after the first oral immunization mice from groups B to H were challenged with H.pylori. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 µl of 3% sodium bicarbonate were given orally to the mice using a stainless steel catheter tube, followed by an oral dose of 5.0×10^9 CFU/ml of Helicobacter pylori. Water and food were returned to the mice after the challenge.

Collection of blood and tissues from mice: Twelve weeks after the first immunization the mice were left overnight without food and subsequently sacrificed for analysis of protection and immune response. The mice were anaesthetized with Metoxyfluorane for terminal cardiac bleeding and prior to sacrifice by cervical dislocation. Under aseptic conditions, spleen and stomach were carefully removed from each mouse and placed on ice in separate sterile containers until further processing. Large and small intestine were obtained for further isolation of the intestinal fluid.

Processing of stomach and measurement of urease activity: The degree of H. pylori colonisation in the mouse stomach was measured by the presence of active urease in the tissue. The Jatrox-test (Röhm-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers' directions. Stomach mucosa was exposed and washed with PBS, half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing the substrate for measurement of urease activity. Absorbance at 550 nm was measured after tubes were incubated for 4 hours at room temperature. The rest of the stomach tissue was stored at -20°C for further treatments. The urease activity values obtained from the stomach of naive mice, which

did not undergo immunization or challenge, were used to create a base line to indicate the absence of H. pylori infection and therefore protection.

Table 1

UreA and UreB expressing S. typhimurium vaccine strains

Strains	Urease Expression	Source
S. typhimurium SL3261	Negative	Hoiseth and Stocker
S. typhimurium SL3262 pYZ97	Constitutive Low	this study
S. typhimurium SL3261::pYZ88pYZ97	High T7-induced expression	this study
S. typhimurium SL3261::pYZ84pYZ97	Medium T7-induced expression	this study
S. typhimurium SL3261::pYZ114pYZ97	Low T7-induced expression	this study

Table 2

Mice groups used for immunization

Group	Immunogen	No. of oral immuniza-tions
A	None	0
B	PBS oral immunization	1
C	S. typhimurium S3261	1
D	S. typhimurium S3261 pYZ97	1
E	S. typhimurium S3261::pYZ88pYZ97	1
F	S. typhimurium S3261::pYZ84pYZ97	1
G	S. typhimurium S3261::pYZ114pYZ97	1
H	Urease B plus cholera toxin	4

Results:

In the control mice (groups B and C) 100% infection with H. pylori was observed. In the mice immunized with recombinant attenuated pathogens (groups D, E, F, G) between 0% and 60% infection (100% to 40% protection) was observed. Immuno-protection did not correlate with humoral anti-UreA and UreB response, suggesting that, in addition to humoral immunity, cellular immunity is critical for protection against H. pylori infection. The results indicate that oral immunization of mice with UreA and UreB delivered by S. typhimurium attenuated strain is effective to induce high levels of protection against H. pylori colonisation.

In the mice immunized with recombinant urease B plus cholera toxin considerably higher levels of urease activity were ob-

served under said experimental conditions than when administering the recombinant attenuated pathogens according to the invention.

The results of the urease test have been illustrated in table 3.

Table 3

Group	Mouse	$E_{550nm, 4h}$	$E_{4h} - E_{control}$	$E_{conc.} * 3$	Dilution
A	1	0,085	-0,022	-0,066	200µl+400µl
A	2	0,091	-0,016	-0,048	200µl+400µl
A	3	0,116	0,009	0,027	200µl+400µl
A	4	0,099	-0,008	-0,024	200µl+400µl
A	5	0,101	-0,006	-0,018	200µl+400µl
Control		0,107	0	0	200µl+400µl
B	1	0,394	0,292	0,876	200µl+400µl
B	2	0,464	0,362	1,086	200µl+400µl
B	3	0,329	0,227	0,681	200µl+400µl
B	4	0,527	0,425	1,275	200µl+400µl
B	5	0,462	0,36	1,08	200µl+400µl
Control		0,102	0	0	200µl+400µl
C	1	0,248	0,145	0,435	200µl+400µl
C	2	0,369	0,266	0,798	200µl+400µl
C	3	0,209	0,106	0,318	200µl+400µl
C	4	0,219	0,116	0,348	200µl+400µl
C	5	0,24	0,137	0,411	200µl+400µl
Control		0,103	0	0	200µl+400µl
D	1	0,143	0,002	0,004	300µl+300µl
D	2	0,156	0,015	0,03	300µl+300µl
D	3	0,142	0,001	0,002	300µl+300µl
D	4	0,114	-0,027	-0,054	300µl+300µl
D	5	0,133	-0,008	-0,016	300µl+300µl
Control		0,141	0	0	300µl+300µl
E	1	0,127	0,027	0,081	200µl+400µl
E	2	0,094	-0,006	-0,018	200µl+400µl
E	3	0,099	-0,001	-0,003	200µl+400µl
E	4	0,161	0,061	0,183	200µl+400µl
E	5	0,198	0,098	0,294	200µl+400µl
Control		0,1	0	0	200µl+400µl
F	1	0,166	0,025	0,05	300µl+300µl
F	2	0,145	0,004	0,008	300µl+300µl
F	3	0,166	0,025	0,05	300µl+300µl
F	4	0,154	0,013	0,026	300µl+300µl
F	5	0,301	0,16	0,32	300µl+300µl
Control		0,141	0	0	300µl+300µl
G	1	0,084	-0,019	-0,057	200µl+400µl
G	2	0,087	-0,016	-0,048	200µl+400µl
G	3	0,269	0,166	0,498	200µl+400µl
G	4	0,085	-0,018	-0,054	200µl+400µl
G	5	0,092	-0,011	-0,033	200µl+400µl
Control		0,103	0	0	200µl+400µl
H	1	0,638	0,531	1,593	200µl+400µl
H	2	0,282	0,175	0,525	200µl+400µl
H	3	0,141	0,034	0,102	200µl+400µl
H	4	0,135	0,028	0,084	200µl+400µl
H	5	0,171	0,064	0,192	200µl+400µl
Control		0,107	0	0	200µl+400µl

Example 3

Construction and molecular analysis of various recombinant *S. typhimurium* strains expressing ureA/ureB subunits.

Description of the S. typhimurium strains used for immunization experiments.

S. typhimurium SL3261(pYZ97) **(construct A):** *S. typhimurium* SL3261 live vaccine vector strain was used as a recipient for the recombinant urease plasmid construct pYZ97.

S. typhimurium SL3261::YZ Series (pYZ97) **(construct B):** These carrier strains are a derivative of *S. typhimurium* SL3261 which has been equipped with the T7 RNA polymerase (T7RNAP) expression cassettes schematically presented in Figure 3. These expression cassettes encode the gene for T7RNAP which is expressed in a 2-phase modus (ON/OFF) as disclosed in a previous invention of Yan et al. ("Two phase system for the production and presentation of foreign antigens in hybrid live vaccines", PCT/EP91/02478). The cassette can be integrated into the chromosome of bacteria and provide the cell in ON-position with optimal amount of T7RNAP for activation of T7RNAP-dependent expression plasmids such as pYZ97.

The principle of the YZ84 cassette is an invertible lambda PL promoter placed on a fragment that is inverted by the phage Mu invertase Gin (Yan & Meyer, 1996, J. Biotechnol. 44:197-201). Dependent on the orientation of the PL promoter either the gin gene (OFF-position) or the T7RNAP gene (ON-position) is expressed. The following regulatory elements have been included in YZ84: (1) The temperature-sensitive cI_{ts} lambda repressor (cI) which represses the PL promoter at 28°C and dissociates at 37°C. (2) The phage fd terminator (fdT) reduces expression of gin gene in order to achieve moderate inversion rates of the PL promoter on the invertible fragment.

The 2-phase expression system enables high expression rates of foreign antigens, such as the urease subunits A and B. It is well known that high expression rates of foreign antigens reduce viability of *Salmonella* carrier thus

diminishing immune response and consequently the protective potential. It was shown that the 2-phase system has a natural competence to improve survival of recombinant *Salmonella* which express large amounts of foreign antigen. In construct B, expression of the *ureA* and *ureB* genes is mainly under the control of the strong T7 promoter resulting in high production of the urease subunits. If the T7RNAP expression cassette is in OFF-position and no T7RNAP is present, the *ureA* and *ureB* genes are constitutively expressed in moderate range by the *Salmonella* promoter.

Analysis of ureA/B subunits produced by the various *S. typhimurium* strains used for immunization experiments.

Salmonella constructs A and B were first analyzed by SDS-polyacrylamide gels for expression of UreA and UreB. The recombinant strains were grown at 37°C in liquid Luria Broth supplemented with 100µg/ml Ampicillin starting from an over night culture. The bacteria were harvested at logarithmic growth phase by centrifugation and the cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard A_{590}=1.0 in all probes. The bacterial suspension was mixed with the same volume of SDS-sample buffer (Sambrook, J. et al. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and boiled for 5 min. 20 µl of suspension were loaded onto two SDS-10 % polyacrylamide gels; one of the gels was stained with Coomassie blue stain and the other was electroblotted onto a nitrocellulose membrane and further processed for immunoblotting. The nitrocellulose membrane carrying the transferred proteins was blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:2000 dilution of rabbit anti-UreB antibody (AK 201) in 5 (w/v)% non-fat milk-TBS was added to the strip and incubated overnight at 4°C. Serum was obtained from rabbit immunised with recombinant urease B subunit purified via affinity chromatography. The membrane was washed three times for 10 min with 0,05 (v/v)%

Tween-20 in PBS, and further incubated in 5 (w/v)% non-fat milk-TBS with goat anti-rabbit IgG antibody horse radish peroxidase conjugate for 45 min at room temperature. After three washes with 0.2 (v/v)% Tween-20 as above, the membrane was developed using the ECL kit (Amersham, Germany) following the recommendations of the suppliers.

Construct A: Proteins of 67 kDa and 30 kDa were observed in the Coomassie stained gel of the whole cell lysate of construct A (*S. typhimurium* strain SL3261(pYZ97); these sizes correlate very well with those of UreB and UreA, respectively. Such proteins were absent in the control lanes containing the *S. typhimurium* SL3261 strain. Immunoblot analysis of the same protein samples using a rabbit anti-UreB antibody confirmed the 67 kDa protein observed in the Coomassie stained gel as UreB. Expression of *ureB* from *S. typhimurium* strain SL3261(pYZ97) was also examined at different phases of growth by incubating at 37°C for 2, 6 and 11 hours, respectively. Expression of *ureB* was observed in all phases of growth including in the stationary phase; although, higher expression was observed at early phases of growth. The results obtained with strain SL3261(pYZ97) indicate that UreA and UreB proteins are non-toxic for *Salmonella* and can be expressed at 37°C at any phase of bacterial growth.

Construct B: Similar analysis were performed with construct B. The comparison of both constructs in SDS-PAGE analysis reveals that construct B is the more efficient producer whilst construct A has moderate expression of *ureA* and *ureB*. In the course of bacterial growth of construct B, the expression of *ureA* and *ureB* is constantly high over a longer time period even without antibiotic selection. This confirms the exceptional productivity of construct B in comparison to construct A.

In summary, our data indicate that UreA and UreB from *H. pylori* can be expressed in *S. typhimurium* without causing adverse effects to the bacteria, and are, therefore, suitable for animal protection experiments when delivered by *Salmonella* carriers.

Plasmid-stability

Plasmid stability is essential to assure stable expression of antigens coded by genes which have been cloned into such plasmids.

In vitro plasmid stability. The ampicillin resistance marker present on plasmid pYZ97 and absent in the plasmidless *S. typhimurium* strain SL3261 was used as an indicator of plasmid stability. *S. typhimurium* strain SL3261 was grown in LB liquid medium at 28°C for up to 100 generations as described previously (Summers, D. K. and D. J. Sherrat. 1984. Cell. 36:1097-1103). Every ten generations, the number of ampicillin resistant CFU was determined from the total number of colony forming units (CFU) of *Salmonella* by plating equal number of bacterial dilutions on plain LB-agar plates and LB-agar plates supplemented with 100 µg/ml ampicillin.

Plasmid stability in vivo. Plasmid stability in vivo was analyzed by examining total CFU and ampicillin resistant CFU from mice spleen, two and seven days after oral infection of mice with 5.0 X10^9 CFU of. *S. typhimurium* SL3261(pYZ97). Mice were orally infected with *Salmonella* as described above. Two days and seven days after infection mice were sacrificed under metoxyfluorane anesthesia, and the spleen was removed aseptically for further processing. The spleen was dissected in small pieces in a petri dish, mixed with 1 ml ice-cold ddH_2O, and passed several times through a 18 gauge needle to suspend the spleen cells. The cell suspension was then plated on LB-agar plates with or without 100 µg/ml ampicillin. Plates were incubated at 37°C overnight and colonies counted the next day.

Plasmid stability *in vivo* was analyzed after infecting mice with one oral dose of 5.0 x 10^9 CFU of *S. typhimurium* SL3261(pYZ97). Mice spleens were taken two and seven days after infection, and plated on LB-agar plates for examination of total CFU and ampicillin resistant CFU. 2.0 x 10^4 ampicillin resistant CFU were isolated from the spleens after 48 h (Table 4). The CFU counts decreased to 56 at 7 days after immunization, but again, all were ampicillin resistant. The

data indicate that plasmid pYZ97 is stable in *Salmonella* under *in vitro* and *in vivo* conditions and is suitable for the evaluation of urease subunits as protective antigens against mouse stomach colonization by *H. pylori*. The low recovery of *Salmonella* strain SL3261 seven days after infection confirms the attenuation of this strain which allows its safe use for delivery of urease into mice.

Table 4

Recovery of *S. typhimurium* SL3261pYZ97 strain from mouse spleens and evaluation of pYZ97 plasmid stability *in vivo*.

Time after infection	Total CFU [a]	Percentage of Amp^r CFU [b]
2 days	$2.0X10^4$	100
7 days	56	100

[a] Number of CFU of *S. typhimuriun* isolated on LB plates without antibiotics from the mouse spleens two and seven days after mice had been orally inoculated with $5.0X10^9$ CFU of *S. typhimuriun* strain SL3261(pYZ97).
[b] Percentage of ampicillin resistant CFU from the total No. of CFU isolated from mouse spleens.

Table 5

Examination of urease activity and streptomycin resistant *H.pylori* in stomach antrum from mice immunized with UreA and UreB-expressing *Salmonella*.

Mice group	No.	Urease activity[a]	CFU[b]
Naive Control Group	5	0.058 ± 0.004	0 ± 0
PBS Control Group	5	0.427 ± 0.059	$2.7X10^3 \pm 1.0X10^3$
SL3261pYZ97[c]	5	0.057 ± 0.006	62.6 ± 97.3

[a] Urease activity is a mean value ± standard deviation.
[b] Determination of CFU of the streptomicin resistant *H. pylori* P76 strain was carried out by plating a section of antrum stomach on serum plates supplemented with 200 µg/ml of streptomycin. *H. pylori* were recognized based on colony morphology, urease activity, and light microscopy examination. Values correspond to CFU ± standard deviation.
[c] Mice immunized with *S. typhimurium* SL3261(pYZ97) expressing *ureA* and *ureB from H. pylori* as described in Materials and Methods.

Example 4

Protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model.

Description of the *Helicobacter pylori* strains used for the experiments

Urease-deficient *H. pylori* P11 strain is a derivative of P1, generated by transposon shuttle mutagenesis using the Tn*Max5* mini-transposon as disclosed in the invention of Haas et al. ("Verfahren zur Identifizierung sekretorischer Gene aus *Helicobacter pylori*"; PCT/EP96/02544). Insertion of Tn*Max5* has been mapped at the 3'-end of the ureA gene resulting in a defect expression of ureA and ureB due to transcriptional coupling of both genes.

Mouse-adapted *H. pylori* P49 strain was originally established by Dr. J. G. Fox (MIT, Boston, MA) from a feline isolate. *H. pylori* P76 strain is a streptomycin-resistant derivative of P49 generated by homologous recombination with chromosomal DNA from streptomycin-resistant *H. pylori* strain NCTC11637 as described by P. Nedenskov-Sorensen (1990, J. Infect. Dis. 161: 365-366).

All *H. pylori* strains were grown at 37°C in a microaerobic atmosphere (5% O_2, 85% N_2, and 10% CO_2) on serum plates (Odenbreit, S. et al. 1996. J. Bacteriol. 178:6960-6967) supplemented with 200 µg/ml of streptomycin when appropriate.

Prophylactic immunization experiments with mice.

Immunization experiments were carried out to test the ability of UreA and B delivered by *Salmonella* to protect mice from stomach colonization by *H. pylori*. In total, 5 independent immunisation experiments have been performed. Each experiment consisted of 5 groups each with 5 mice: (1) **naive control group** was mice neither immunized with *Salmonella* nor challenged with wild type *H. pylori* P49 or the streptomycin resistant derivative strain P76; (2) **PBS control group** was non-immunized mice that received PBS and were challenged

orally with *H. pylori*; (3) ***Salmonella* control group** was mice immunized with attenuated *S. typhimurium* SL3261 strain alone and challenged with *H. pylori*; and (5) the **vaccine group** was the mice immunized with appropriate recombinant *S. typhimurium* construct (A + B) expressing UreA and UreB and challenged with *H. pylori*.

Prior to immunizations, mice were left overnight without solid food and 4 hours without water. 100 µl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Immediately after stomach neutralization, mice from the **PBS control group** received 100 µl PBS, and mice from the ***Salmonella* control group** and ***Salmonella* vaccine group**, received 5.0 X 10^9 CFU of *S. typhimurium* strain SL3261 and the various recombinant constructs, respectively, in a total volume of 100 µl. Water and food were returned to the mice after immunization.

Four weeks after the oral immunization, mice from the **PBS control-**, ***Salmonella* control-** and **vaccine-groups** were challenged with 1.0X10^9 CFU of *H. pylori*. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 µl of 3% sodium bicarbonate were given orally to mice using a stainless steel catheter tube, followed by an oral dose of 1.0 X 10^9 CFU/ml of *H. pylori* strains P49 or P76 . Water and food were returned to mice after challenge.

Example 5

Immunological analyses of protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model

Collection of blood and intestinal fluid from mice for serological analyses.

Antibody responses were evaluated from all mice using serum and intestinal fluid. 150 µl blood were collected retro-orbitally before immunization and three weeks after immunization, before *Helicobacter* infection. The final bleeding was carried out 11 weeks after *Salmonella*

immunization (6 weeks after challenge infection) by terminal cardiac puncture under metoxyfluorane anesthesia. The small intestines were taken from mice at the end of experiment and processed as described before (Elson, C. O. et al 1984. J. Immunol. Meth. 67:101-108) with minor modifications. Briefly, the content of intestines was removed by passing 2 ml of 50mM EDTA pH 7.5 (Riedel) containing 0,1mg/ml Soybean trypsin inhibitor (Sigma). The volume was adjusted to 5 ml with 0.15 M NaCl. The samples were vortexed vigorously, centrifuged 10 min at 2,500 rpm (Heraeus, Germany), and supernatant supplemented with 50 µl of 100 mM phenylmethylsulfonylfluoride (PMSF) (Serva) in 95% ethanol, followed by centrifugation at 13,000rpm for 20 min at 4°C (Hermes). Supernatants were supplemented with 50 µl of 100 mM PMSF and 50 µl of 2% sodium azide (Merck) and left on ice 15 min before addition of 250 µl of 7% bovine serum albumine (Biomol). The samples were frozen at -20°C until further use.

Analysis of anti-urease antibodies in mouse sera and intestinal mucosa by ELISA.

Oral immunization with *Salmonella* is known to elicit IgA antibody responses. The IgA response against urease subunits in mice immunized with *S. typhimurium* construct A + B and in control mice was assessed by ELISA. A soluble extract of *H. pylori* P1 and its urease-deficient mutant derivative strain P11 was prepared in phosphate-buffer-saline by sonicating five times with a sonifier (Branson , Danbury, Conn.) at 5 sec intervals (35 % pulses) for 45 sec. This suspension was centrifuged at 13,000 rpm (Heraeus, Germany) for 10 min at 4°C to remove intact cells. The supernatant was used as antigen after determination of the protein content using the BioRad kit. 96-well microtiter plates (Nunc, Germany) were coated with 50 µl aliquot of 50 µg/ml of antigen in sodium carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C. The wells were blocked with 1.0 (w/v)% non-fat milk in Tris-buffer-saline (TBS) for 45 min at room temperature and washed three times with TBS-0.05% Tween-20. The assays, which were performed in triplicate, used 50 µl of serum or gut washing

diluted 1:100 or 1:2 respectively in 0.5 (w/v)% non-fat milk-TBS added to the wells and left overnight at 4°C. The wells were then washed three times with TBS-0.05% Tween 20, and a 1:3000 dilution of a goat anti-mouse IgA horse-radish peroxidase-conjugate (Sigma) was added to all wells and incubated overnight at 4°C. The color reaction was developed by incubation at 37°C for 30 min with an orthophenylendiamine substrate in sodium acetate buffer and hydrogen peroxide. The reaction was stopped with 10 N H_2SO_4 and the A_{492} was determined in an ELISA reader (Digiscan, Asys Hitech GmbH, Austria).

Mucosal antibodies: (**Construct A**) Intestinal fluid was taken from each sacrificed mouse at the end of the experiment (six weeks after the *H. pylori* challenge) and tested for the presence of anti-urease antibodies by using total cell extracts of *H. pylori* wild type (P1) and urease deficient mutant strains (P11). As shown in Fig. 4, the IgA antibody response against the wild type *H. pylori* extract was around 10-fold higher in immunized mice versus non-immunized or naive mice. The mucosal IgA antibody response against the urease-deficient *H. pylori* mutant was very low in all groups of mice indicating that most of the intestinal IgA antibody response in immunized mice was directed against urease.

Serum antibodies: (**Construct A**) The levels of serum IgA antibodies against a wild type and an urease-deficient *H. pylori* were examined prior to immunization, 3 weeks after immunization (before challenge) and 10 weeks after immunization (6 weeks after challenge with *H. pylori*). As shown in Fig. 5 panel A, the levels of anti-wild type *H. pylori* antibodies in mice immunized with urease-expressing *S. typhimurium* construct A were ~20-fold higher at three weeks and 34-fold higher ten weeks after immunization with respect to the pre-immune serum. The serum IgA antibody response against the urease-deficient *H. pylori* strain at 3 and 10 weeks was low in all groups of mice including the mice immunized with *Salmonella* construct A (Fig. 5, panel B), indicating that most of the IgA antibody response in immunized mice is directed against the urease subunits. Low serum

antibody responses against wild type *H. pylori* were also observed at ten weeks in non-immunized mice suggesting that the *H. pylori* challenge given three weeks earlier was enough to induce a specific antibody response in these mice. The IgA response to wild type *H. pylori* in mice immunized for three weeks with *S. typhimurium* SL3261 (*Salmonella* control group) increased moderately, which may be explained by the presence of antigens in *Salmonella* that are able to induce cross-reacting antibodies against *H. pylori*. In contrast, the antibody response against the urease-deficient *H. pylori* strain in immunized mice was as low as the antibody response of non-immunized mice (Fig 5, panel B). This result suggests that most of the antibody response observed in immunized mice was against urease. Low antibody response against the urease-negative mutant was observed in the 10 weeks sera from mice given PBS or immunized with *S. typhimurium* SL3261, suggesting that the antibody response observed is due to the specific immune response against the *H. pylori* antigens given to these mice three weeks earlier during challenge. A low antibody response against the urease-deficient *H. pylori* strain was observed at three weeks in mice immunized with *Salmonella* either expressing or not expressing urease, but was absent in the mice given PBS. This confirms the presence of cross-reacting epitopes between proteins from *Salmonella* and *H. pylori*, respectively. **(Construct B):** The serological analysis of mice immunized with the construct B series achieved similar results indicating that higher production of antigen by recombinant *Salmonella* does not significantly increase antibody response.

Analysis of anti-urease antibodies in mouse sera by immunoblotting.

Expression of UreA and UreB from *S. typhimurium* necessary for the induction of mice specific immune response against *H. pylori* was analyzed. Identification of *in vivo* expression of UreA and UreB was carried out by looking for anti-UreA and anti-UreB antibodies in serum of mice immunized with *Salmonella* construct A and control mice. *H. pylori* whole-cell

antigens were prepared from the wild type *H. pylori* strain P1. Bacteria were recovered from 3 serum plates, resuspended in PBS, and harvested by 10 min centrifugation at 5,000 g. The cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard A_{590}=1.0 in all probes. The bacterial suspension was mixed with same volume of SDS-sample buffer (Sambrook, 1989) and boiled for 5 min. 20 µl Pellet were loaded onto a SDS-10% polyacrylamide gel. The proteins were electro-blotted onto a nitrocellulose membrane and cut into strips which were blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:80 dilution of mouse serum in 5 (w/v)% non-fat milk-TBS was added to the strips and incubated overnight at 4°C. Sera was obtained from mice non-immunized and immunized with *Salmonella*. After three washes, the strips were incubated with a goat anti-mouse IgG horse-radish peroxidase conjugate (Sigma) diluted 1:3000 in 5 (w/v)% non-fat milk-TBS. The ECL chemi-luminescence detection kit (Amersham, Germany) was used for development of blots according to the supplier' s directions.

Serum from immunized and non-immunized mice was obtained 3 weeks after immunization prior to the challenge with *H. pylori* and tested against whole-cell lysates of the wild type *H. pylori* P1 strain expressing UreA and UreB. Proteins of 67 kDa and 30 kDa in size, corresponding to UreB and UreA, respectively, were recognized by serum from immunized mice immunized with construct A. These bands were not observed in strips tested with serum from non-immunized mice or mice immunized with *Salmonella* only, suggesting that urease expressed by the *Salmonella* vaccine strain was able to induce a specific antibody response against both UreA and UreB of a wild type *H. pylori* strain. Similar results were obtained with construct B.

Example 6

Determination of *H. pylori* colonisation in mice pretreated with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model

Processing of stomach and measurement of urease activity.

Urease-test: Analysis of protection against stomach colonization by *H. pylori* was performed by testing for urease activity in the antral portion of the mouse stomach. Measurement of urease activity is a very reliable, sensitive and specific method to test for the presence of *H. pylori* infection (NIH consensus development on *Helicobacter pylori* in peptic ulcer disease. 1994. *Helicobacter pylori* in peptic ulcer disease. JAMA. 272:65) and is routinely used in clinical settings (Kawanishi, M., S. et al 1995. J. Gastroenterol. 30:16-20; Kamija, S. et al 1993. Eur. J. Epidemiol. 9:450-452; Conti-Nibali, S. et al 1990. Am. J. Gastroenterol. 85:1573-1575) and in animal research (Gottfried, M. R. et al 1990. Am. J. Gastroenterol. 85:813-818). The Jatrox-test (Röhn-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers directions. Eleven weeks after immunization with *Salmonella*, mice were sacrificed and the stomach was carefully removed under aseptic conditions. The stomach was placed in ice-cold PBS in an sterile container, and the mucosa was exposed by making an incision along the minor curvature with a sterile blade. The stomach was rinsed with PBS to remove food residues and dissected to isolate the antral region from the corpus region. Half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing 500 µl of the urease substrate from Jatrox-test. The stomach sample was incubated 4 h at room temperature and the absorbance at 550 nm (A_{550}) measured. The urease activity values obtained from the stomach of naive mice, which did not undergo immunization or challenge, were used to determine the baseline. The baseline corresponded to the average urease activity value from five naive mice stomachs tested plus two times the standard deviation of this average. Urease activity values higher than the baseline were considered *H. pylori* colonization positive and values below the baseline were

considered *H. pylori* colonization negative.

Cultivation experiment: The left portion of the antral region of stomachs obtained from mice challenged with the streptomycin resistant *H. pylori* strain P76 were plated on serum plates supplemented with 200 μg/ml of streptomycin and incubated under standard conditions. After three days incubation, bacteria were identified as *H. pylori* based on colony morphology, microscopic examination, and urease activity. The number of colony forming units (CFU) of *H. pylori* grown on plates was determined from each mouse stomach sample.

Urease test (Construct A vs. B): Mice immunized with ~$5.0X10^9$ CFU of *Salmonella* and challenged with $1.0X10^9$ CFU of *H. pylori* strain P49, as well as control mice, were sacrificed under anesthesia and a section of the antral region of the stomach was taken for measurement of urease activity. As shown in Fig. 6, 100% of the mice immunized with UreA and B delivered by *Salmonella* construct A had urease activity below the baseline, indicating the absence of *H. pylori* colonisation. In contrast, 100 % of the non-immunized mice (PBS) and the mice immunized with *S. typhimurium* strain SL3261 alone, had urease activity measurements far above the baseline indicating stomach colonization by *H. pylori*. The naive group of mice, which did not undergo immunization or challenge, was used to set the baseline of urease activity.

Salmonella of the construct B-series had urease activity values above the baseline indicating stomach colonization by *H. pylori* challenge strain. However, the urease activities within this group were lower as in the controls suggesting a partial protection status of mice immunized with the Salmonella construct B series (Figure 6). Both Salmonella constructs, A and B, mediate similar antibody response but differed in expression of ureA and ureB. We conclude from this that the quantity of expressed urease antigen is relevant to gain optimal protection.

Construct A: To correlate stomach colonization by *H. pylori* with urease activity a new protection experiment was

performed by immunizing mice orally with *Salmonella* construct A and challenging them with the streptomycin resistant *H. pylori* P76 strain. Urease activity values correlated with the number of CFU of *H. pylori* identified. In two of the five mice immunized with urease-expressing *Salmonella*, no *H. pylori* CFU were detected and the average number of CFU in all five immunized mice was only 62. In contrast, the number of CFU in non-immunized mice was 2,737, which corresponds to 44-fold higher colonization. These data indicate that mice immunized with urease-expressing *Salmonella* were able to eliminate or significantly decrease colonizing *H. pylori* from mouse stomachs.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Max-Planck-Gesellschaft zur Foerderung der Wissenschaften e.V. Berlin
(B) STREET: Hofgartenstr. 2
(C) CITY: Muenchen
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): 80539

(ii) TITLE OF INVENTION: Helicobacter pylori live vaccine

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1557 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helicobacter pylori

(vii) IMMEDIATE SOURCE:
(B) CLONE: alpB

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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   144
Met Thr Phe Gly Tyr Glu Leu Gly Gln Val Val Gln Gln Val Lys Asn
         35                  40                  45

CCG GGT AAA ATC AAA GCC GAA GAA TTA GCC GGC TTG TTA AAC TCT ACC
   192
Pro Gly Lys Ile Lys Ala Glu Glu Leu Ala Gly Leu Leu Asn Ser Thr
     50                  55                  60

ACA ACA AAC AAC ACC AAT ATC AAT ATT GCA GGC ACA GGA GGC AAT GTC
   240
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 65                  70                  75                  80

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   288
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                85                  90                  95

GAT TTG TAT CCC ACT TTG AAC ACT AGT AAT ATC ACA CAA TGT GGC ACT
   336
Asp Leu Tyr Pro Thr Leu Asn Thr Ser Asn Ile Thr Gln Cys Gly Thr
            100                 105                 110

ACT AAT AGT GGT AGT AGT AGT AGT GGT GGT GGT GCG GCC ACA GCC GCT
   384
Thr Asn Ser Gly Ser Ser Ser Ser Gly Gly Gly Ala Ala Thr Ala Ala
        115                 120                 125

GCT ACT ACT AGC AAT AAG CCT TGT TTC CAA GGT AAC CTG GAT CTT TAT
   432
Ala Thr Thr Ser Asn Lys Pro Cys Phe Gln Gly Asn Leu Asp Leu Tyr
    130                 135                 140

AGA AAA ATG GTT GAC TCT ATC AAA ACT TTG AGT CAA AAC ATC AGC AAG
   480
Arg Lys Met Val Asp Ser Ile Lys Thr Leu Ser Gln Asn Ile Ser Lys
145                 150                 155                 160

AAT ATC TTT CAA GGC AAC AAC AAC ACC ACG AGC CAA AAT CTC TCC AAC
   528
Asn Ile Phe Gln Gly Asn Asn Asn Thr Thr Ser Gln Asn Leu Ser Asn
                165                 170                 175

CAG CTC AGT GAG CTT AAC ACC GCT AGC GTT TAT TTG ACT TAC ATG AAC
   576
Gln Leu Ser Glu Leu Asn Thr Ala Ser Val Tyr Leu Thr Tyr Met Asn
            180                 185                 190
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TCG TTC TTA AAC GCC AAT AAC CAA GCG GGT GGG ATT TTT CAA AAC AAC
   624
Ser Phe Leu Asn Ala Asn Asn Gln Ala Gly Gly Ile Phe Gln Asn Asn
        195                 200                 205

ACT AAT CAA GCT TAT GGA AAT GGG GTT ACC GCT CAA CAA ATC GCT TAT
   672
Thr Asn Gln Ala Tyr Gly Asn Gly Val Thr Ala Gln Gln Ile Ala Tyr
    210                 215                 220

ATC CTA AAG CAA GCT TCA ATC ACT ATG GGG CCA AGC GGT GAT AGC GGT
   720
Ile Leu Lys Gln Ala Ser Ile Thr Met Gly Pro Ser Gly Asp Ser Gly
225                 230                 235                 240

GCT GCC GCA GCG TTT TTG GAT GCC GCT TTA GCG CAA CAT GTT TTC AAC
   768
Ala Ala Ala Ala Phe Leu Asp Ala Ala Leu Ala Gln His Val Phe Asn
                245                 250                 255

TCC GCT AAC GCC GGG AAC GAT TTG AGC GCT AAG GAA TTC ACT AGC TTG
   816
Ser Ala Asn Ala Gly Asn Asp Leu Ser Ala Lys Glu Phe Thr Ser Leu
            260                 265                 270

GTG CAA AAT ATC GTC AAT AAT TCT CAA AAC GCT TTA ACG CTA GCC AAC
   864
Val Gln Asn Ile Val Asn Asn Ser Gln Asn Ala Leu Thr Leu Ala Asn
        275                 280                 285

AAC GCT AAC ATC AGC AAT TCA ACA GGC TAT CAA GTG AGC TAT GGC GGG
   912
Asn Ala Asn Ile Ser Asn Ser Thr Gly Tyr Gln Val Ser Tyr Gly Gly
    290                 295                 300

AAT ATT GAT CAA GCG CGA TCT ACC CAA CTA TTA AAC AAC ACC ACA AAC
   960
Asn Ile Asp Gln Ala Arg Ser Thr Gln Leu Leu Asn Asn Thr Thr Asn
305                 310                 315                 320

ACT TTG GCT AAA GTT AGC GCT TTG AAT AAC GAG CTT AAA GCT AAC CCA
  1008
Thr Leu Ala Lys Val Ser Ala Leu Asn Asn Glu Leu Lys Ala Asn Pro
                325                 330                 335

TGG CTT GGG AAT TTT GCC GCC GGT AAC AGC TCT CAA GTG AAT GCG TTT
  1056
Trp Leu Gly Asn Phe Ala Ala Gly Asn Ser Ser Gln Val Asn Ala Phe
            340                 345                 350

AAC GGG TTT ATC ACT AAA ATC GGT TAC AAG CAA TTC TTT GGG GAA AAC
  1104
Asn Gly Phe Ile Thr Lys Ile Gly Tyr Lys Gln Phe Phe Gly Glu Asn
        355                 360                 365
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AAG AAT GTG GGC TTA CGC TAC TAC GGC TTC TTC AGC TAT AAC GGC GCG
  1152
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    370                 375                 380

GGC GTG GGT AAT GGC CCT ACT TAC AAT CAA GTC AAT TTG CTC ACT TAT
  1200
Gly Val Gly Asn Gly Pro Thr Tyr Asn Gln Val Asn Leu Leu Thr Tyr
385                 390                 395                 400

GGG GTG GGG ACT GAT GTG CTT TAC AAT GTG TTT AGC CGC TCT TTT GGT
  1248
Gly Val Gly Thr Asp Val Leu Tyr Asn Val Phe Ser Arg Ser Phe Gly
                405                 410                 415

AGT AGG AGT CTT AAT GCG GGC TTC TTT GGG GGG ATC CAA CTC GCA GGG
  1296
Ser Arg Ser Leu Asn Ala Gly Phe Phe Gly Gly Ile Gln Leu Ala Gly
            420                 425                 430

GAT ACT TAC ATC AGC ACG CTA AGA AAC AGC TCT CAG CTT GCG AGC AGA
  1344
Asp Thr Tyr Ile Ser Thr Leu Arg Asn Ser Ser Gln Leu Ala Ser Arg
        435                 440                 445

CCT ACA GCG ACG AAA TTC CAA TTC TTG TTT GAT GTG GGC TTA CGC ATG
  1392
Pro Thr Ala Thr Lys Phe Gln Phe Leu Phe Asp Val Gly Leu Arg Met
    450                 455                 460

AAC TTT GGT ATC TTG AAA AAA GAC TTG AAA AGC CAT AAC CAG CAT TCT
  1440
Asn Phe Gly Ile Leu Lys Lys Asp Leu Lys Ser His Asn Gln His Ser
465                 470                 475                 480

ATA GAA ATC GGT GTG CAA ATC CCT ACG ATT TAC AAC ACT TAC TAT AAA
  1488
Ile Glu Ile Gly Val Gln Ile Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
                485                 490                 495

GCT GGC GGT GCT GAA GTG AAA TAC TTC CGC CCT TAT AGC GTG TAT TGG
  1536
Ala Gly Gly Ala Glu Val Lys Tyr Phe Arg Pro Tyr Ser Val Tyr Trp
            500                 505                 510

GTC TAT GGC TAC GCC TTC TAA
  1557
Val Tyr Gly Tyr Ala Phe
        515
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 518 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Thr Gln Ser Gln Lys Val Arg Phe Leu Ala Pro Leu Ser Leu Ala
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35 40 45

Pro Gly Lys Ile Lys Ala Glu Glu Leu Ala Gly Leu Leu Asn Ser Thr
50 55 60

Thr Thr Asn Asn Thr Asn Ile Asn Ile Ala Gly Thr Gly Gly Asn Val
65 70 75 80

Ala Gly Thr Leu Gly Asn Leu Phe Met Asn Gln Leu Gly Asn Leu Ile
85 90 95

Asp Leu Tyr Pro Thr Leu Asn Thr Ser Asn Ile Thr Gln Cys Gly Thr
100 105 110

Thr Asn Ser Gly Ser Ser Ser Ser Gly Gly Gly Ala Ala Thr Ala Ala
115 120 125

Ala Thr Thr Ser Asn Lys Pro Cys Phe Gln Gly Asn Leu Asp Leu Tyr
130 135 140

Arg Lys Met Val Asp Ser Ile Lys Thr Leu Ser Gln Asn Ile Ser Lys
145 150 155 160

Asn Ile Phe Gln Gly Asn Asn Asn Thr Thr Ser Gln Asn Leu Ser Asn
165 170 175

Gln Leu Ser Glu Leu Asn Thr Ala Ser Val Tyr Leu Thr Tyr Met Asn
180 185 190

Ser Phe Leu Asn Ala Asn Asn Gln Ala Gly Gly Ile Phe Gln Asn Asn
195 200 205

Thr Asn Gln Ala Tyr Gly Asn Gly Val Thr Ala Gln Gln Ile Ala Tyr
210 215 220

Ile Leu Lys Gln Ala Ser Ile Thr Met Gly Pro Ser Gly Asp Ser Gly
225 230 235 240

Ala Ala Ala Ala Phe Leu Asp Ala Ala Leu Ala Gln His Val Phe Asn
245 250 255

Ser Ala Asn Ala Gly Asn Asp Leu Ser Ala Lys Glu Phe Thr Ser Leu
260 265 270

Val Gln Asn Ile Val Asn Asn Ser Gln Asn Ala Leu Thr Leu Ala Asn
275 280 285

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290 295 300

Asn Ile Asp Gln Ala Arg Ser Thr Gln Leu Leu Asn Asn Thr Thr Asn
305 310 315 320

Thr Leu Ala Lys Val Ser Ala Leu Asn Asn Glu Leu Lys Ala Asn Pro
325 330 335

Trp Leu Gly Asn Phe Ala Ala Gly Asn Ser Ser Gln Val Asn Ala Phe
340 345 350

Asn Gly Phe Ile Thr Lys Ile Gly Tyr Lys Gln Phe Phe Gly Glu Asn
355 360 365

Lys Asn Val Gly Leu Arg Tyr Tyr Gly Phe Phe Ser Tyr Asn Gly Ala
370 375 380

Gly Val Gly Asn Gly Pro Thr Tyr Asn Gln Val Asn Leu Leu Thr Tyr
385 390 395 400

Gly Val Gly Thr Asp Val Leu Tyr Asn Val Phe Ser Arg Ser Phe Gly
405 410 415

Ser Arg Ser Leu Asn Ala Gly Phe Phe Gly Gly Ile Gln Leu Ala Gly
420 425 430

Asp Thr Tyr Ile Ser Thr Leu Arg Asn Ser Ser Gln Leu Ala Ser Arg
435 440 445

Pro Thr Ala Thr Lys Phe Gln Phe Leu Phe Asp Val Gly Leu Arg Met
450 455 460

Asn Phe Gly Ile Leu Lys Lys Asp Leu Lys Ser His Asn Gln His Ser
465 470 475 480

Ile Glu Ile Gly Val Gln Ile Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
485 490 495

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Val Tyr Gly Tyr Ala Phe
515

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1557 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Helicobacter pylori

(vii) IMMEDIATE SOURCE:
(B) CLONE: alpA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1554

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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    96
Ser Ile Ser Tyr Ala Glu Asp Asp Gly Gly Phe Phe Thr Val Gly Tyr
535                 540                 545                 550

CAG CTC GGG CAA GTC ATG CAA GAT GTC CAA AAC CCA GGC GGC GCT AAA
   144
Gln Leu Gly Gln Val Met Gln Asp Val Gln Asn Pro Gly Gly Ala Lys
                555                 560                 565

AGC GAC GAA CTC GCC AGA GAG CTT AAC GCT GAT GTA ACG AAC AAC ATT
   192
Ser Asp Glu Leu Ala Arg Glu Leu Asn Ala Asp Val Thr Asn Asn Ile
            570                 575                 580

TTA AAC AAC AAC ACC GGA GGC AAC ATC GCA GGG GCG TTG AGT AAC GCT
   240
Leu Asn Asn Asn Thr Gly Gly Asn Ile Ala Gly Ala Leu Ser Asn Ala
        585                 590                 595

TTC TCC CAA TAC CTT TAT TCG CTT TTA GGG GCT TAC CCC ACA AAA CTC
   288
Phe Ser Gln Tyr Leu Tyr Ser Leu Leu Gly Ala Tyr Pro Thr Lys Leu
    600                 605                 610

AAT GGT AGC GAT GTG TCT GCG AAC GCT CTT TTA AGT GGT GCG GTA GGC
   336
Asn Gly Ser Asp Val Ser Ala Asn Ala Leu Leu Ser Gly Ala Val Gly
615                 620                 625                 630
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TCT GGG ACT TGT GCG GCT GCA GGG ACG GCT GGT GGC ACT TCT CTT AAC
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                635                 640                 645

ACT CAA AGC ACT TGC ACC GTT GCG GGC TAT TAC TGG CTC CCT AGC TTG
   432
Thr Gln Ser Thr Cys Thr Val Ala Gly Tyr Tyr Trp Leu Pro Ser Leu
            650                 655                 660

ACT GAC AGG ATT TTA AGC ACG ATC GGC AGC CAG ACT AAC TAC GGC ACG
   480
Thr Asp Arg Ile Leu Ser Thr Ile Gly Ser Gln Thr Asn Tyr Gly Thr
        665                 670                 675

AAC ACC AAT TTC CCC AAC ATG CAA CAA CAG CTC ACC TAC TTG AAT GCG
   528
Asn Thr Asn Phe Pro Asn Met Gln Gln Gln Leu Thr Tyr Leu Asn Ala
    680                 685                 690

GGG AAT GTG TTT TTT AAT GCG ATG AAT AAG GCT TTA GAG AAT AAG AAT
   576
Gly Asn Val Phe Phe Asn Ala Met Asn Lys Ala Leu Glu Asn Lys Asn
695                 700                 705                 710

GGA ACT AGT AGT GCT AGT GGA ACT AGT GGT GCG ACT GGT TCA GAT GGT
   624
Gly Thr Ser Ser Ala Ser Gly Thr Ser Gly Ala Thr Gly Ser Asp Gly
                715                 720                 725

CAA ACT TAC TCC ACA CAA GCT ATC CAA TAC CTT CAA GGC CAA CAA AAT
   672
Gln Thr Tyr Ser Thr Gln Ala Ile Gln Tyr Leu Gln Gly Gln Gln Asn
            730                 735                 740

ATC TTA AAT AAC GCA GCG AAC TTG CTC AAG CAA GAT GAA TTG CTC TTA
   720
Ile Leu Asn Asn Ala Ala Asn Leu Leu Lys Gln Asp Glu Leu Leu Leu
        745                 750                 755

GAA GCT TTC AAC TCT GCC GTA GCC GCC AAC ATT GGG AAT AAG GAA TTC
   768
Glu Ala Phe Asn Ser Ala Val Ala Ala Asn Ile Gly Asn Lys Glu Phe
    760                 765                 770

AAT TCA GCC GCT TTT ACA GGT TTG GTG CAA GGC ATT ATT GAT CAA TCT
   816
Asn Ser Ala Ala Phe Thr Gly Leu Val Gln Gly Ile Ile Asp Gln Ser
775                 780                 785                 790

CAA GCG GTT TAT AAC GAG CTC ACT AAA AAC ACC ATT AGC GGG AGT GCG
   864
Gln Ala Val Tyr Asn Glu Leu Thr Lys Asn Thr Ile Ser Gly Ser Ala
                795                 800                 805
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GTT ATT AGC GCT GGG ATA AAC TCC AAC CAA GCT AAC GCT GTG CAA GGG
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           810                 815                 820

CGC GCT AGT CAG CTC CCT AAC GCT CTT TAT AAC GCG CAA GTA ACT TTG
   960
Arg Ala Ser Gln Leu Pro Asn Ala Leu Tyr Asn Ala Gln Val Thr Leu
       825                 830                 835

GAT AAA ATC AAT GCG CTC AAT AAT CAA GTG AGA AGC ATG CCT TAC TTG
  1008
Asp Lys Ile Asn Ala Leu Asn Asn Gln Val Arg Ser Met Pro Tyr Leu
    840                 845                 850

CCC CAA TTC AGA GCC GGG AAC AGC CGT TCA ACG AAT ATT TTA AAC GGG
  1056
Pro Gln Phe Arg Ala Gly Asn Ser Arg Ser Thr Asn Ile Leu Asn Gly
855                 860                 865                 870

TTT TAC ACC AAA ATA GGC TAT AAG CAA TTC TTC GGG AAG AAA AGG AAT
  1104
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                875                 880                 885

ATC GGT TTG CGC TAT TAT GGT TTC TTT TCT TAT AAC GGA GCG AGC GTG
  1152
Ile Gly Leu Arg Tyr Tyr Gly Phe Phe Ser Tyr Asn Gly Ala Ser Val
           890                 895                 900

GGC TTT AGA TCC ACT CAA AAT AAT GTA GGG TTA TAC ACT TAT GGG GTG
  1200
Gly Phe Arg Ser Thr Gln Asn Asn Val Gly Leu Tyr Thr Tyr Gly Val
       905                 910                 915

GGG ACT GAT GTG TTG TAT AAC ATC TTT AGC CGC TCC TAT CAA AAC CGC
  1248
Gly Thr Asp Val Leu Tyr Asn Ile Phe Ser Arg Ser Tyr Gln Asn Arg
    920                 925                 930

TCT GTG GAT ATG GGC TTT TTT AGC GGT ATC CAA TTA GCC GGT GAG ACC
  1296
Ser Val Asp Met Gly Phe Phe Ser Gly Ile Gln Leu Ala Gly Glu Thr
935                 940                 945                 950

TTC CAA TCC ACG CTC AGA GAT GAC CCC AAT GTG AAA TTG CAT GGG AAA
  1344
Phe Gln Ser Thr Leu Arg Asp Asp Pro Asn Val Lys Leu His Gly Lys
                955                 960                 965

ATC AAT AAC ACG CAC TTC CAG TTC CTC TTT GAC TTC GGT ATG AGG ATG
  1392
Ile Asn Asn Thr His Phe Gln Phe Leu Phe Asp Phe Gly Met Arg Met
           970                 975                 980
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AAC TTC GGT AAG TTG GAC GGG AAA TCC AAC CGC CAC AAC CAG CAC ACG
  1440
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        985                 990                 995

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  1488
Val Glu Phe Gly Val Val Val Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
    1000                1005                1010

TCA GCA GGG ACT ACC GTG AAG TAT TTC CGT CCT TAT AGC GTT TAT TGG
  1536
Ser Ala Gly Thr Thr Val Lys Tyr Phe Arg Pro Tyr Ser Val Tyr Trp
1015                1020                1025                1030

TCT TAT GGG TAT TCA TTC TAA
  1557
Ser Tyr Gly Tyr Ser Phe
                1035
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 518 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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Met Ile Lys Lys Asn Arg Thr Leu Phe Leu Ser Leu Ala Leu Cys Ala
  1               5                  10                  15

Ser Ile Ser Tyr Ala Glu Asp Asp Gly Gly Phe Phe Thr Val Gly Tyr
            20                  25                  30

Gln Leu Gly Gln Val Met Gln Asp Val Gln Asn Pro Gly Gly Ala Lys
        35                  40                  45

Ser Asp Glu Leu Ala Arg Glu Leu Asn Ala Asp Val Thr Asn Asn Ile
    50                  55                  60

Leu Asn Asn Asn Thr Gly Gly Asn Ile Ala Gly Ala Leu Ser Asn Ala
65                  70                  75                  80

Phe Ser Gln Tyr Leu Tyr Ser Leu Leu Gly Ala Tyr Pro Thr Lys Leu
                85                  90                  95

Asn Gly Ser Asp Val Ser Ala Asn Ala Leu Leu Ser Gly Ala Val Gly
            100                 105                 110

Ser Gly Thr Cys Ala Ala Ala Gly Thr Ala Gly Gly Thr Ser Leu Asn
        115                 120                 125
```

Thr Gln Ser Thr Cys Thr Val Ala Gly Tyr Tyr Trp Leu Pro Ser Leu
130 135 140

Thr Asp Arg Ile Leu Ser Thr Ile Gly Ser Gln Thr Asn Tyr Gly Thr
145 150 155 160

Asn Thr Asn Phe Pro Asn Met Gln Gln Gln Leu Thr Tyr Leu Asn Ala
165 170 175

Gly Asn Val Phe Phe Asn Ala Met Asn Lys Ala Leu Glu Asn Lys Asn
180 185 190

Gly Thr Ser Ser Ala Ser Gly Thr Ser Gly Ala Thr Gly Ser Asp Gly
195 200 205

Gln Thr Tyr Ser Thr Gln Ala Ile Gln Tyr Leu Gln Gly Gln Gln Asn
210 215 220

Ile Leu Asn Asn Ala Ala Asn Leu Leu Lys Gln Asp Glu Leu Leu Leu
225 230 235 240

Glu Ala Phe Asn Ser Ala Val Ala Ala Asn Ile Gly Asn Lys Glu Phe
245 250 255

Asn Ser Ala Ala Phe Thr Gly Leu Val Gln Gly Ile Ile Asp Gln Ser
260 265 270

Gln Ala Val Tyr Asn Glu Leu Thr Lys Asn Thr Ile Ser Gly Ser Ala
275 280 285

Val Ile Ser Ala Gly Ile Asn Ser Asn Gln Ala Asn Ala Val Gln Gly
290 295 300

Arg Ala Ser Gln Leu Pro Asn Ala Leu Tyr Asn Ala Gln Val Thr Leu
305 310 315 320

Asp Lys Ile Asn Ala Leu Asn Asn Gln Val Arg Ser Met Pro Tyr Leu
325 330 335

Pro Gln Phe Arg Ala Gly Asn Ser Arg Ser Thr Asn Ile Leu Asn Gly
340 345 350

Phe Tyr Thr Lys Ile Gly Tyr Lys Gln Phe Phe Gly Lys Lys Arg Asn
355 360 365

Ile Gly Leu Arg Tyr Tyr Gly Phe Phe Ser Tyr Asn Gly Ala Ser Val
370 375 380

Gly Phe Arg Ser Thr Gln Asn Asn Val Gly Leu Tyr Thr Tyr Gly Val
385 390 395 400

Gly Thr Asp Val Leu Tyr Asn Ile Phe Ser Arg Ser Tyr Gln Asn Arg
405 410 415

Ser Val Asp Met Gly Phe Phe Ser Gly Ile Gln Leu Ala Gly Glu Thr
420 425 430

Phe Gln Ser Thr Leu Arg Asp Asp Pro Asn Val Lys Leu His Gly Lys
435 440 445

Ile Asn Asn Thr His Phe Gln Phe Leu Phe Asp Phe Gly Met Arg Met
450 455 460

Asn Phe Gly Lys Leu Asp Gly Lys Ser Asn Arg His Asn Gln His Thr
465 470 475 480

Val Glu Phe Gly Val Val Val Pro Thr Ile Tyr Asn Thr Tyr Tyr Lys
485 490 495

Ser Ala Gly Thr Thr Val Lys Tyr Phe Arg Pro Tyr Ser Val Tyr Trp
500 505 510

Ser Tyr Gly Tyr Ser Phe
515

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 656 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:567..656

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGATCTATGA ATCTATGATA TCAACACTCT TTTTGATAAA TTTTCTCGAG GTACCGAGCT
60

TGAGGCATCA AATAAAACGA AAGGCTCAGT CGAAAGACTG GGCCTTTCGT TTTATCTGTT
120

GTTTGTCGGT GAACGCTCTC CTGAGTAGGA CAAATCCGCC GGGAGCGGAT TTGAACGTTG
180

CGAAGCAACG GCCCGGAGGG TGGCGGGCAG GACGCCCGCC ATAAACTGCC ACAAGCTCGG
240

TACCGTTGAT CTTCCTATGG TGCACTCTCA GTACAATCTG CTCTGATGCG CTACGTGACT
300

GGGTCATGGC TGCGCCCCGA CACCCGCCAA CACCCGCTGA CGCGCCCTGA CGGGCTTGTC

360

TGCTCCCGGC ATCCGCTTAC AGACAAGCTG TGACCGTCTC CGGGAGCTGC ATGTGTCAGA
420

GGTTTTCACC GTCATCACCG AAACGCGCGA GGCCCAGCGC TTCGAACTTC TGATAGACTT
480

CGAAATTAAT ACGACTCACT ATAGGGAGAC CACAACGGTT TCCCTCTAGA AATAATTTTG
540

TTTAACTTTA AGAAGGAGAT ATACAT ATG AAA CTG ACT CCC AAA GAG TTA GAC
593
Met Lys Leu Thr Pro Lys Glu Leu Asp
520 525

AAG TTG ATG CTC CAC TAC GCT GGA GAA TTG GCT AAA AAA CGC AAA GAA
641
Lys Leu Met Leu His Tyr Ala Gly Glu Leu Ala Lys Lys Arg Lys Glu
530 535 540

AAA GGC ATT AAG CTT
656
Lys Gly Ile Lys Leu
545

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Leu Thr Pro Lys Glu Leu Asp Lys Leu Met Leu His Tyr Ala
1 5 10 15

Gly Glu Leu Ala Lys Lys Arg Lys Glu Lys Gly Ile Lys Leu
20 25 30

Patent Claims

1. A recombinant attenuated microbial pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable to express said nucleic acid molecule or capable to cause the expression of said nucleic acid molecule in a target cell.

2. The pathogen according to claim 1, which is an enterobacterial cell, especially a Salmonella cell.

3. The pathogen according to claim 1 or 2, which is a Salmonella aro mutant cell.

4. The pathogen according to any of claims 1-3, wherein the Helicobacter antigen is urease, a urease subunit, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

5. The pathogen according to any one of claims 1-3, wherein the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive fragment thereof, or a peptide mimotope thereof.

6. The pathogen according to any one of claims 1-3 and 5, wherein the Helicobacter antigen is selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive fragments thereof, or a peptide mimotope thereof.

7. The pathogen according to any one of claims 1-6, wherein said nucleic acid molecule encoding a Helicobacter antigen is capable to be expressed phase variably.

8. The pathogen according to claim 7,

wherein said nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal which is substantially inactive in the pathogen and which is capable to be activated by a nucleic acid reorganization caused by a nucleic acid reorganization mechanism in the pathogen.

9. The pathogen according to claim 8, wherein the expression signal is a bacteriophage promoter, and the activation is caused by a DNA reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

10. The pathogen according to any one of claims 1-9, further comprising at least one second nucleic acid molecule encoding an immunomodulatory polypeptide, wherein said pathogen is capable to express said second nucleic acid molecule.

11. Pharmaceutical composition comprising as an active agent a recombinant attenuated pathogen according to any one of claims 1-10, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants.

12. Composition according to claim 11, which is a living vaccine, which is suitable for administration to a mucosal surface or via the parenteral route.

13. A method for the preparation of a living vaccine comprising formulating an attenuated pathogen according to any one of claims 1-10 in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

14. A method for preparing a recombinant attenuated pathogen according to any one of claims 1-10, comprising the steps:

a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein a recombinant attenuated pathogen is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell, and

b) cultivating said recombinant attenuated pathogen under suitable conditions.

15. The method according to claim 14, wherein said nucleic acid molecule encoding a Helicobacter antigen is located on an extrachromosomal plasmid or inserted in the chromosome.

16. A method for identifying Helicobacter antigens, which raise a protective immune response in a mammalian host, comprising the steps of:

a) providing an expression gene bank of Helicobacter in an attenuated pathogen and

b) screening the clones of the gene bank for their ability to confer protective immunity against a Helicobacter infection in a mammalian host.

Figure 1
Genetic map of the expression plasmid pYZ97

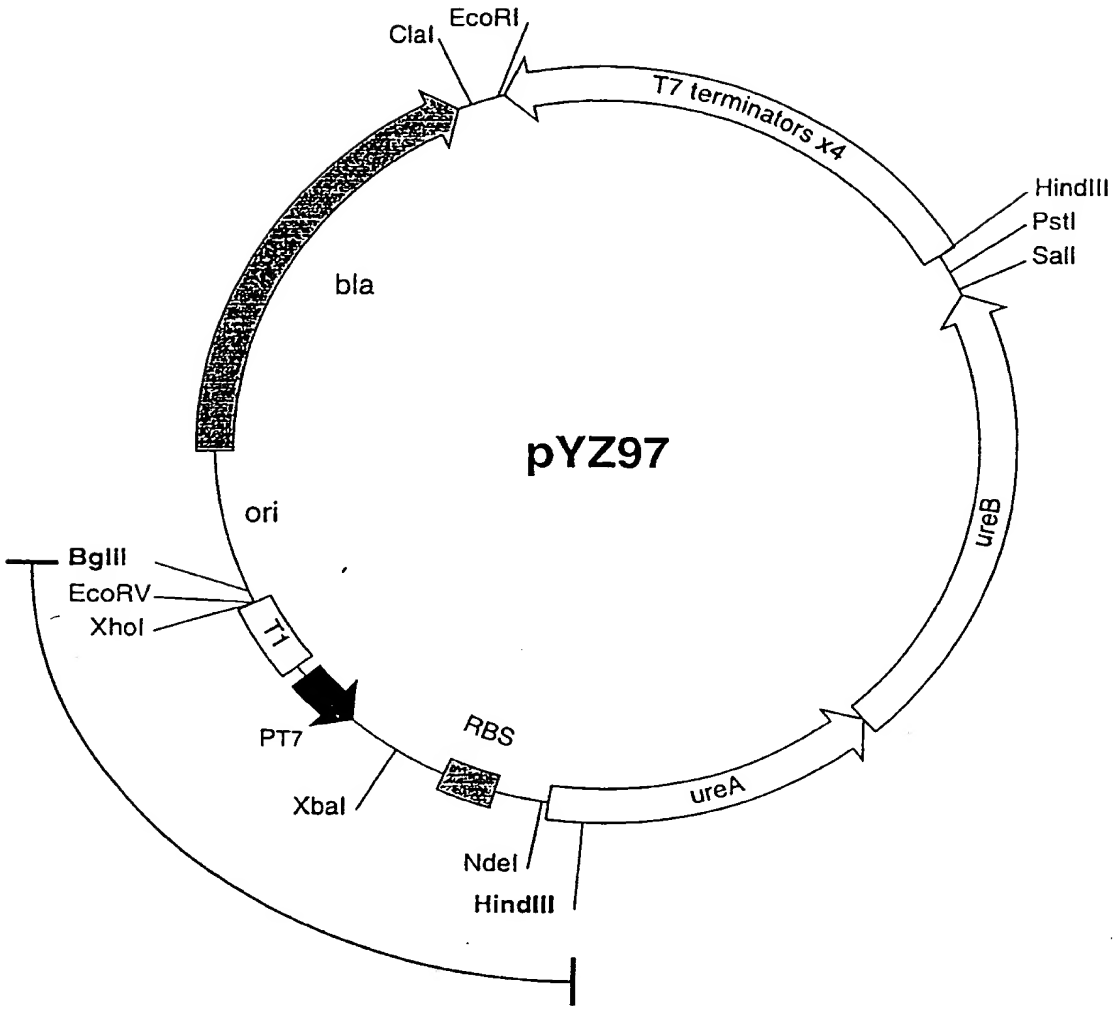


Figure 2

Nucleotide sequence of the transcriptional regulators for urease expression on plasmid pYZ97

```
1   AG ATC TAT GAA TCT ATG ATA TCA ACA CTC TTT TTG ATA AAT TTT CTC GAG GTA CCG AGC
    BglII              EcoRV                                 XhoI
                                    rrnB T1
60  TTG AGG CAT CAA ATA AAA CGA AAG GCT CAG TCG AAA GAC TGG GCC TTT CGT TTT ATC TGT

120 TGT TTG TCG GTG AAC GCT CTC CTG AGT AGG ACA AAT CCG CCG GGA GCG GAT TTG AAC GTT
                                                              -35
180 GCG AAG CAA CGG CCC GGA GGG TGG CGG GCA GGA CGC CCG CCA TAA ACT GCC ACA AGC TCG
                  -10
240 GTA CCG TTG ATC TTC CTA TGG TGC ACT CTC AGT ACA ATC TGC TCT GAT GCG CTA CGT GAC

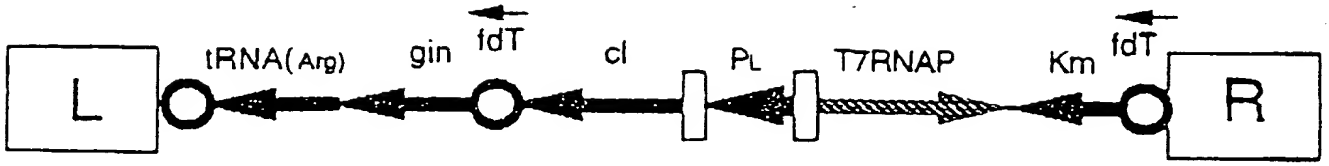
300 TGG GTC ATG GCT GCG CCC CGA CAC CCG CCA ACA CCC GCT GAC GCG CCC TGA CGG GCT TGT

360 CTG CTC CCG GCA TCC GCT TAC AGA CAA GCT GTG ACC GTC TCC GGG AGC TGC ATG TGT CAG

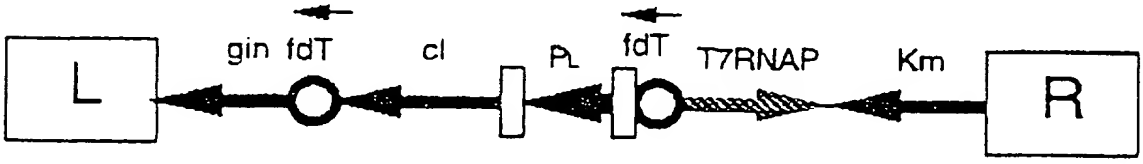
420 AGG TTT TCA CCG TCA TCA CCG AAA CGC GCG AGG CCC AGC GCT TCG AAC TTC TGA TAG ACT
                        PT7
480 TCG AAA TTA ATA CGA CTC ACT ATA GGG AGA CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT
                                                             XbaI
                      RBS                        down stream box
                                  YZ019
540 GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AAA CTG ACT CCC AAA GAG TTA GAC AAG TTG
                                        Met Lys Leu Thr Pro Lys Glu Leu Asp Lys Leu

600 ATG CTC CAC TAC GCT GGA GAA TTG GCT AAA AAA CGC AAA GAA AAA GGC ATT AAG CTT
    Met Leu His Tyr Ala Gly Glu Leu Ala Lys Lys Arg Lys Glu Lys Gly Ile Lys Leu
```

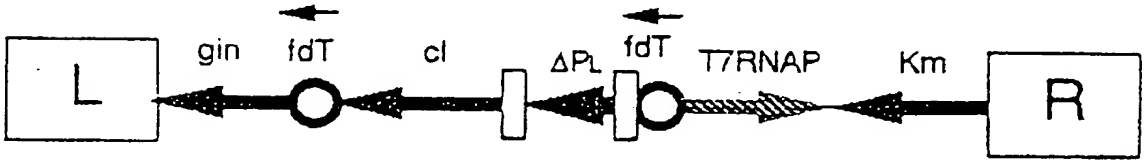
Figure 3



pYZ88 (high expression)



pYZ84 (medium expression)



pYZ114 (low expression)

Figure 4

ELISA for anti-*H. pylori* IgA antibodies in intestinal fluids of vaccinated mice

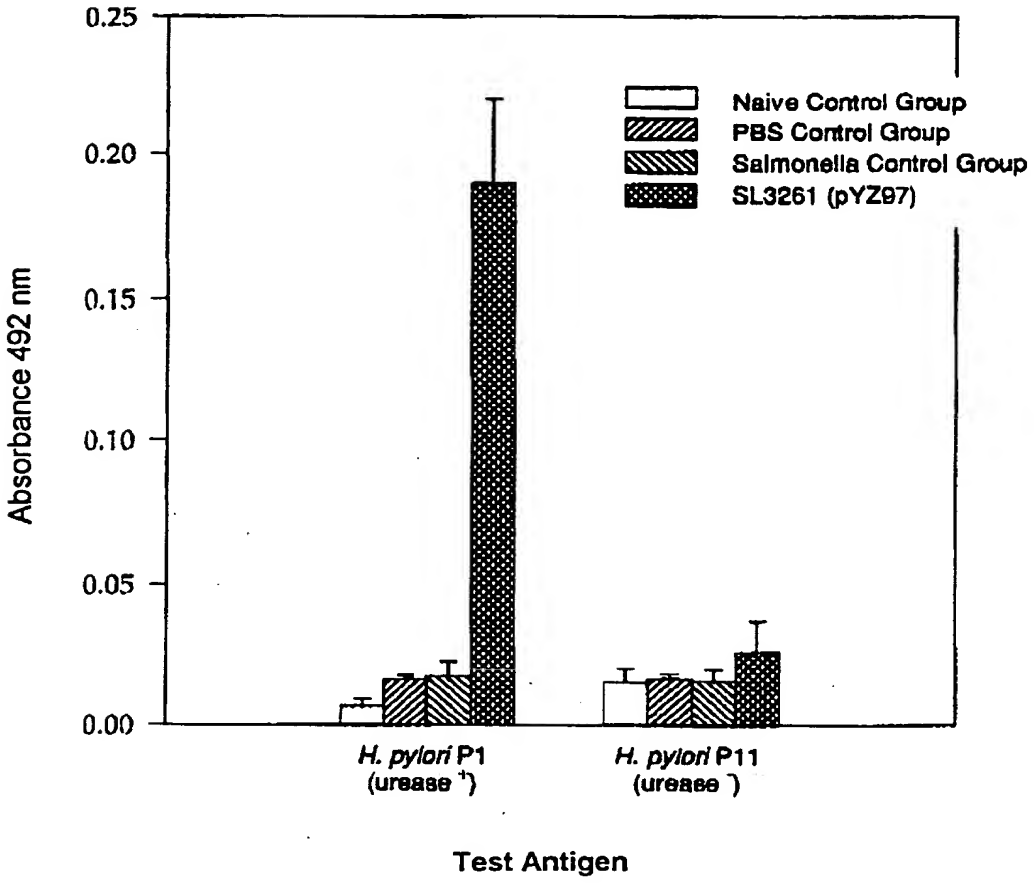


Figure 5

ELISA for anti-*H. pylori* IgA antibodies in serum of vaccinated mice

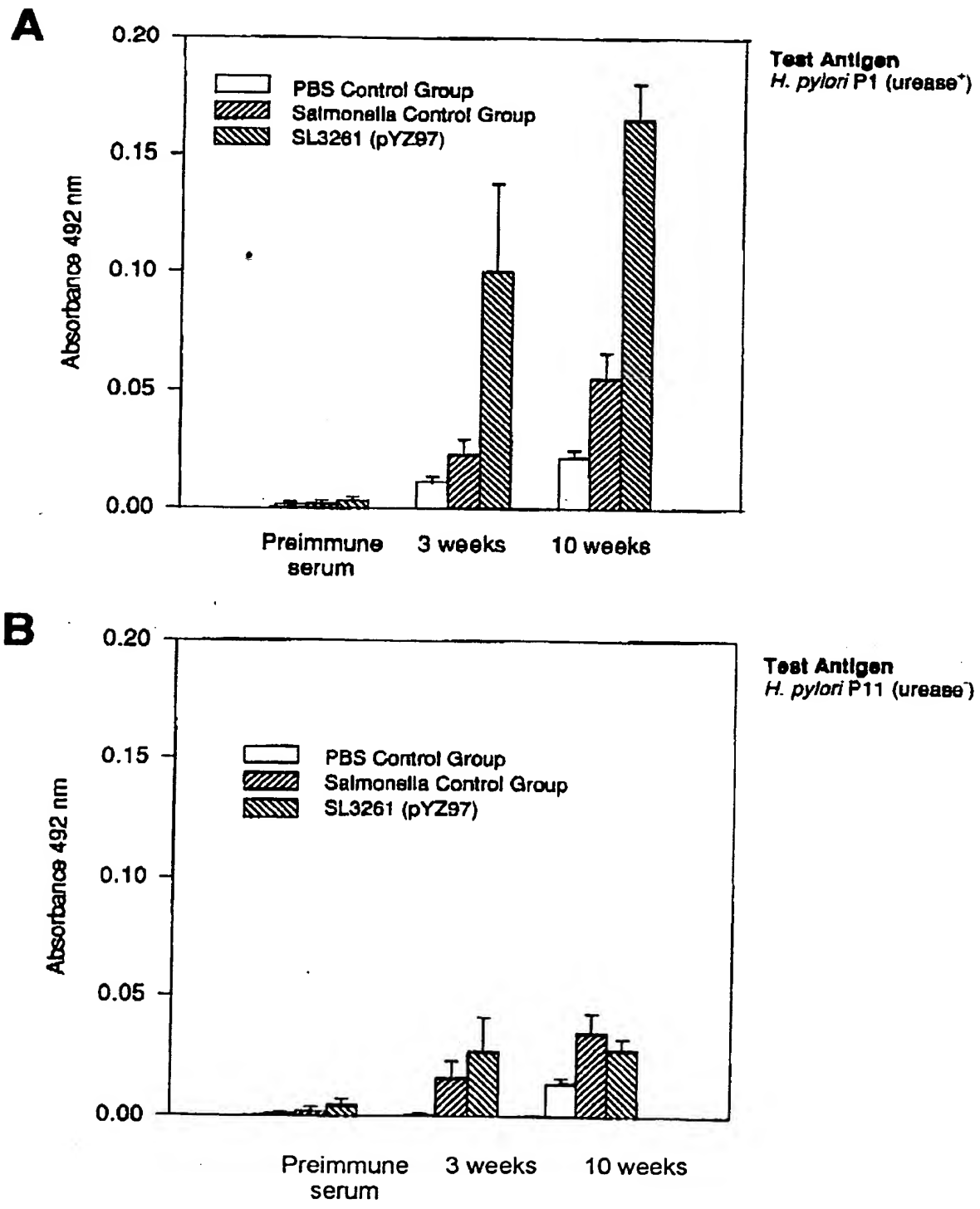
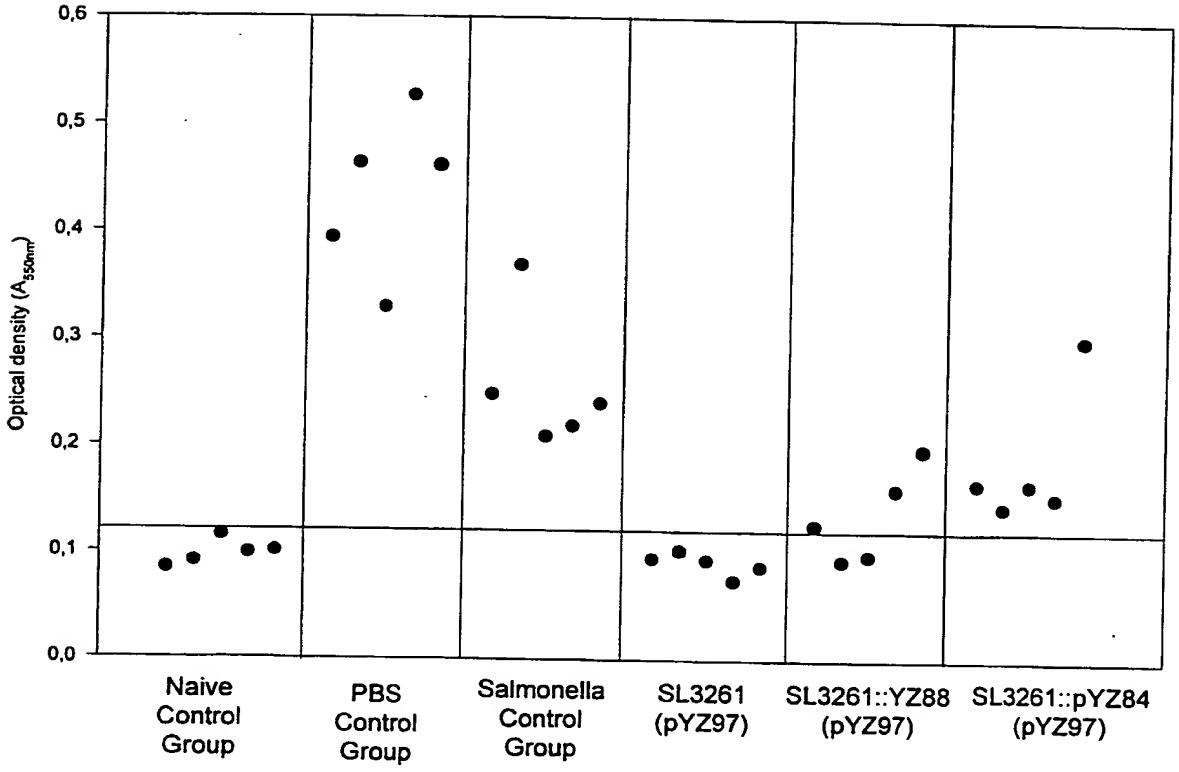


Figure 6

Urease activity in stomach tissue of vaccinated mice after *H. pylori* challenge.



INTERNATIONAL SEARCH REPORT

Inter. ional Application No
PCT/EP 97/04744

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/205 C07K16/12 C12N15/10 C12N15/31 C12N15/74
A61K39/112

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A.A. LINDBERG : "The history of live bacterial vaccines" DEVELOPMENTS IN BIOLOGICAL STANDARDIZATION, vol. 84, 1995, BASEL CH, pages 211-219, XP002051591 see the whole document, especially the summary	1-3,11, 13-15
X	WO 93 07273 A (INSTITUT PASTEUR) 15 April 1993 see page 3 - page 4 see page 24 - page 25	1,4

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 1998	03.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

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INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/EP 97/04744

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 93 10214 A (G. GEORGIOU) 27 May 1993 see page 11, line 20 - line 30 see page 13, line 4 - line 21 see page 23, line 16 - page 25, line 29 see page 26, line 15 - line 25	1
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 97/04744

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